BICYCLIC AZOALKANES AS PROBES AND SENSORS IN SUPRAMOLECULAR CHEMISTRY

by

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School of Engineering and Science
Dedicated to my beloved parents
“You’ve got to work hard, you’ve got to work hard.
If you want anything at all”
Depeche mode
ABSTRACT

This thesis is focused on the investigation of the complexation of several bicyclic azoalkanes by cyclodextrins and calixarenes, selected as models of host molecules. The distinguished characteristics, such as water-solubility, spherical shape, microenvironmental sensitivity, and most importantly, the extremely long-lived fluorescence of the singlet excited-state of the 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO), and its homologues and derivatives (Scheme 1) make azoalkanes suitable to be used as guests and molecular probes in supramolecular chemistry. Regarding potential applications, azoalkanes are very useful in the study of solution structures of inclusion complexes, chiral resolution, the kinetics of diastereomeric supramolecular association, the design of chemical sensors, and as enzyme mimics.

Scheme 1. Bicyclic azoalkanes investigated in this work.

Assignments of solution structures of cyclodextrin (CD) inclusion complexes of azoalkanes 1-6 were made by induced circular dichroism (ICD) based on the orientation-intensity ICD rules of Harata and Kodaka. With respect to these rules, two examples of complex structures with lateral and axial alignment of the guests in the β-CD cavity displaying positive ICD signals were suggested (Scheme 2).

Scheme 2. Complex structures predicted according to ICD rules.

The differential solubility of diasteromeric capsules self-assembled from chiral guest molecules (7) and two natural β-CDs (Scheme 3) was introduced as a novel concept for chiral
resolution. From a racemic mixture, the (−)-enantiomer can be enriched in the precipitate and the (+)-enantiomer in the supernatant solution. Determined as 30%, the enantiomeric enrichment in the precipitate could be greatly improved by magnifying the ratio of the binding constant product \(K_1K_2\). Also, a significant effect on the association kinetics with the derivative methylated-\(\beta\)-CD was found, which is ascribed to a ca. 20% faster complexation for the (−)-enantiomer.

**Scheme 3. Chiral resolution through self-assembled encapsulation.**

Molecular recognition studies with the water-soluble \(p\)-sulfonatocalix[4]arene (CX4) (Scheme 4) showed the highest binding constants for the guests 1, 2 and 4 (ca. \(10^3\) M\(^{-1}\)) in comparison to previously investigated noncharged organic molecules (ca. 10-100 M\(^{-1}\)). The pronounced spherical shape affinity of the CX4 conical cavity was deemed responsible for the strong binding. Moreover, the driving force for shape matching between host and guest molecules was sufficiently strong to prefer the inclusion of the highly hydrophilic bicyclic azo residue over the hydrophobic phenyl group in 6. This revealed that shape complementarity was more important, as a recognition element, than hydrophobic interactions in the CX4 binding process, which was contrary to the binding preference of \(\beta\)-CD (Scheme 2).

**Scheme 4. Sensor system based on the fluorescence regeneration of DBO.**

One motivation behind supramolecular chemistry was to design chemical systems capable of mimicking enzyme functions. In this approach, the alteration of the chemical reactivity of guests by formation of inclusion complexes was exemplified. Upon complexation by CX4, an enhanced basicity for azoalkanes 1, 4 and 8 was observed and led to large p\(K_a\) shifts (ca. 2 units) through hydrophobic and electrostatic interactions (Scheme 4).
The fluorescence regeneration of DBO from the CX4•DBO inclusion complex (Scheme 4), upon addition of analytes, allowed us to design a sensor system that could operate over a large pH range. Through displacement assays, CX4 was found to be a selective receptor for cholines rather than carnitines (see structures below), due to strong electrostatic effects. By this approach, targeting the binding of metal ions was reported for the first time. Further improvement with the CX4•9 system enhanced the detection performance of fluorescence recovery in the micromolar range, and allowed the use of the sensitive time-resolved detection technique. This provided ideal conditions for the development of label-free supra-biomolecular tandem assays for bioassay applications.

Finally, supramolecular metallo-enzyme models exhibiting metal-substrate binding interaction involved in biocatalytic processes were designed by means of non-covalent interactions between zinc (II) ion and geometrically controlled CX4 inclusion complexes of DBO (equatorial) and its dialkylated derivative 8 (axial), as shown in Scheme 5. Under neutral conditions, in opposite to the metal-induced release observed for the azoalkane 8, the near-UV shift as well as the fluorescence quenching of DBO clearly provided evidence for coordination to Zn$^{2+}$ ion, thereby forming a stable ternary complex. Further evidence was obtained by the complexation-induced $^1$H-NMR shifts of the guest protons as well as by the X-ray structure of the CX4•DBO•Zn$^{2+}$ complex (Scheme 5). Interestingly, a remarkable case of Zn$^{2+}$ coordination upon displacement of the proton of the complexed protonated DBO was observed in acidic solution.

**Scheme 5.** Supramolecular Zinc metallo-enzyme models mimicking structural specificity-dependence of metal-substrate binding interaction
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The present thesis was carried out during the period of 2000-2005 in the Department of Physical Chemistry at the University of Basel (Switzerland), and at the International University Bremen (Germany).

The scientific achievements obtained during my Ph.D. research are reflected in 12 papers and one patent. Among them, nine are already published, and three others are in preparation as well as one patent is submitted. The work, which I describe in this thesis, concerned with "Bicyclic Azoalkanes as Probes and Sensors in Supramolecular Chemistry", is based on nine manuscripts, and one patent which are denoted with an asterisk.

In Journals:

1) Exploiting Long-Lived Molecular Fluorescence

2) Induced Circular Dichroism and Structural Assignment of the Cyclodextrin Inclusion Complexes of Bicyclic Azoalkanes*

3) Chiral Resolution Through Precipitation of Diastereomeric Capsules in the Form 2:1 β-Cyclodextrin-Guest Complexes*

4) Chiral Discrimination in the Complexation of Heptakis-(2,6-di-O-methyl)-β-Cyclodextrin with 2,3-Diazabicyclo[2.2.2]oct-2-ene Derivatives*


6) Spherical Shape Complementarity as an Overriding Motif in the Molecular Recognition of Noncharged Organic Guests by p-Sulfonatocalix[4]arene: Complexation of Bicyclic Azoalkanes*

7) Effect of Temperature, Cholesterol Content, Antioxidant Structure on the Mobility of Vitamin E Constituents in Biomembrane Models Studied by Laterally Diffusion-Controlled Fluorescence Quenching
8) Fluorescence Regeneration as Signaling Principle for Choline and Carnitine Binding by \textit{p}-Tetrasulfonatocalix\[4\]arene: A Refined Supramolecular Sensor System Based on a Fluorescent Azoalkane*

9) Analysis of Host-Assisted Guest Protonation Exemplified for \textit{p}-Sulfonatocalix\[4\]arene – Towards Enzyme-Mimetic \textit{p}K\textsubscript{a} Shifts*

10) Label-Free Supra-Biomolecular Tandem Assays*

\textit{In Preparation:}

11) Chiral Discrimination in the Kinetics of Ternary Complexation of Natural, Heptakis-(2,6-di-O-methyl)- and Heptakis-(2,3,6-tri-O-methyl)-\(\beta\)-Cyclodextrins with 2,3-Diazabicyclo[2.2.2]oct-2-ene Derivatives Assessed by Global Analysis of Fluorescence Lifetime Decays
Bakirci, H.; Nau, W. M.

12) Calixarene-Based Zinc Metallo-Enzyme Models Exhibiting Structural Specificity-Dependence of Metal-Substrate Binding Interaction*
Bakirci, H.; Koner, A. L.; Nau, W. M.

13) Label-Free Supra-Biomolecular Tandem Assays*
Bakirci, H.; Hennig A.; Nau, W. M. (manuscript).
ATTENTED CONFERENCES

1) A Novel Azo Chromophore for Probing Supramolecular Kinetics and Constructing Photonic Devices
   Zhang, X.; Bakirci, H.; Márquez, C.; Pischel, U.; Nau, W. M.
   National Research Program 47 "Supramolecular Functional Materials"
   June 30, 2000, Bern, Switzerland

2) The Fluorazophore Approach: Applications in Supramolecular Chemistry and Biology
   Bakirci, H.; Pischel, U.; Zhang, X.; Nau, W. M.
   National Research Program 47 "Supramolecular Functional Materials"
   October 23, 2001, Bern, Switzerland

3) Understanding Host-Guest Systems
   Bakirci, H.; Márquez, C.; Nau, W. M.
   National Research Program 47 "Supramolecular Functional Materials"
   July 4, 2003, Bern, Switzerland
   – This poster was selected as one of the three best presentations of the NRP47 meeting –

4) Complexation of Water-Soluble Calix[4]arenes with 2,3-Diazabicyclo[2.2.2]oct-2-ene
   Bakirci, H.; Schatz, J.; Nau, W. M.
   7th International Conference on Calixarenes
   August 13-16, 2003, Vancouver, Canada

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   Spring School in Supramolecular Chemistry, National Research Program 47
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6) The 2,3-Diazabicyclo[2.2.2]oct-2-ene•Calixarene Fluorescent Sensor System
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Chapter 1

INTRODUCTION
1.1 Supramolecular Chemistry

Supramolecular chemistry has become one of the most interdisciplinary and fastest growing areas of research in chemistry offering an effective interface between biology and materials science. The term of supramolecular chemistry was coined, among others, by Jean-Marie Lehn in his study of inclusion compounds and cryptands. In 1987, Jean-Marie Lehn, Donald Cram and Charles Pedersen were jointly awarded the Nobel Prize for their pioneering work. Nowadays, supramolecular chemistry has been defined as "the chemistry of molecular assemblies and of the intermolecular bond" or "the chemistry beyond molecules", where noncovalent forces and spatial arrangements between interacting individual molecules play a major role in the formation of these entities. Ion-paring (electrostatic interactions), hydrophobic interactions, hydrogen bonds, π-π stacking, cation-π interactions, and van der Waals forces are typical examples of the intermolecular forces that dominate supramolecular chemistry. Compared to covalent bonds, these interactions are weaker and usually reversible. Because of their dynamic nature, these interactions are responsible for most biological processes such as cellular recognition, the assembling of protein complexes, antibody-antigen complexes, highly selective enzymatic catalysis, self-recognition of the complementarity base-pairs leading to the self-assembly of the double helix, etc.

Scheme 1-1. Some typical examples of host systems.
Supramolecular chemistry has evolved from efforts to mimic weak noncovalent interactions and the phenomenon of molecular recognition ubiquitous in living systems. The characterizing feature of supramolecular chemistry is that carefully designed artificial receptor (host) molecules are capable of recognizing substrate (guest) species, forming supramolecular or host-guest complexes of well-defined structures and functions through noncovalent interactions. Since Pederson’s discovery of crown ethers and their abilities to bind strongly metal ions in 1967, host-guest chemistry has exploded and contributed to the emergence of various types of supramolecular hosts, e.g., cyclodextrins, calixarenes, cucurbiturils, cavitands, dendrimers, and others (Scheme 1-1). Their binding properties such as selectivity and binding strength towards a wide range of organic and inorganic substrates have been extensively investigated involving many experimental methods. Starting from enzyme mimics, the concept of host-guest complexation has been extended to pharmacology, chromatography and materials science.

1.2 Macrocyclic Host Molecules: Cyclodextrins and Calixarenes

Important to the progress in supramolecular host-guest chemistry has been the design of macrocyclic host molecules able to form inclusion complexes with guest molecules. Association between host and guest molecules are usually based on simultaneous noncovalent interactions between single binding sites. For host-guest complexation studies, several parameters like host size, charge, solubility, and others have to be taken into account according to the properties of the target molecule. The optimal stability between host and guest is reached if the complementarity of binding sites between the host and the guest matches to each other.

Our work on host-guest complexation is concerned with cyclodextrins (CDs) and calixarenes (Scheme 1-2), which are two representative examples of water-soluble supramolecular macrocyclic hosts. Among the CD family, the three natural CDs as well as the derivative heptakis-(2,6-di-O-methyl)-β-CD (DMe-β-CD) have been investigated. Regarding the wide class of calixarenes, p-sulfonatocalix[4]arene (CX4) has been selected for our studies. Both host molecules are large hollow organic molecules with well-defined cavities which are accessible by smaller guest molecules.

**Cyclodextrins.** CDs were discovered by Villiers in 1891. They are produced from degradation of starch by cyclodextrin glucanotransferase enzyme (CGTase). CDs are chemically stable and water-soluble cyclic oligosaccharides made up of α(1-4)-linked D-glucopyranose units. Their molecular shape is similar to a hollow truncated cone, which contains a hydrophilic outer surface and a relatively hydrophobic central cavity, into which organic molecules can be incorporated. The most common CDs are represented by α-, β-, and γ-CDs, which respectively are composed of 6, 7, or 8 monomers leading to different cavity sizes. The inner diameter of the hydrophobic cavity is approximately 4.7-5.3, 6.0-6.5 and 7.5-8.3 Å for α-CD, β-CD, and γ-CD, respectively. The interior cavity is covered by the hydrogen atoms linked to the corresponding C-3 and C-5 and by the glycosidic O-4 oxygen atoms, while the outer surface is surrounded by the secondary O-2 and O-3 hydroxyl groups located at the wider “upper” rim, and by the primary O-6 hydroxyl groups building up the narrower “lower” rim (Scheme 1-2). The macrocyclic structure is stabilized by O-2 (n)…O-3 (n-1) intramolecular hydrogen bonds between two adjacent syn-oriented α-D-glucopyranose residues.

Due to their interesting molecular recognition ability and physicochemical characteristics, CD-based host-guest complexation has found numerous applications in the development of fluorescent chemical sensors, in catalysis, photochemistry and photophysics, analytical chemistry, as well as in the biotechnology, food, cosmetic and pharmaceutical industries. Their application in pharmaceutical drug development is known to bring about an enhancement of solubility, chemical stability and bioavailability for drugs. Since CDs are themselves chiral, they can form a diastereomeric pair of inclusion complexes with each enantiomer of a racemate. Consequently, the use of CDs as chiral selectors in capillary electrophoresis, stationary or mobile-phase components in high performance liquid chromatography as well as NMR shift reagents has been widely exploited. Further, chemical modifications to the parent CDs, in order to enhance their solubility, molecular recognition ability, and selectivity, e.g., through derivatization of the hydroxyl groups at C-2, C-3 and C-6 position of D-glucopyranose, resulted in a broadening of applications in the area of bioprocessing, medicine and analytical chemistry.
Chapter 1

Introduction

Design of CD-based host-guest systems for specific applications require an improved understanding and control of the solution dynamic behaviour exhibited by these systems. Therefore, CDs have been investigated in several thermodynamic and kinetic studies of host-guest complexation. Thermodynamic (enthalpy, entropy and binding constant) and kinetic (entry and exit rate) data relevant for understanding dynamic aspects of the inclusion phenomena have been collected by means of UV,^{26,27} NMR,^{22} fluorescence,^{28,29} and microcalorimetry.^{30,31} In addition, the structural elucidation of such host-guest complexes is also an important prerequisite to establish structure-reactivity relationships related to interaction mode, and molecular size and shape, which play an important role in the molecular recognition process.

The solution (not solid-state) structures of CD complexes have been mainly studied by NMR^{22} and induced circular dichroism (ICD).^{32} Since the pioneering work of Demarco and Thakkar^33 on the study of CD complexes, the contribution of NMR^{22} has been highly relevant and enlightening for the assignment of CD complexes based on the changes in chemical shift for the CD H-3 and H-5 protons through guest inclusion as well as the close proximity interactions between the CD and guest protons, e.g., NOE and ROESY effects. Compared to NMR, ICD has been recognized as a suitable sensitive tool for structural assignment of the relative alignment of chromophoric guests^44,35 with \( \pi,\pi^* \) transition inside the CD chiral cavity. Recent studies on CD complexes with aliphatic azoalkanes^{28,29,36-38} have demonstrated the validity of this method to assign the co-conformations of \( n,\pi^* \) azo chromophores inside CDs on the basis of the established orientation-intensity ICD rules of Harata^34 and Kodaka.^35 These co-conformations have been even confirmed by computational calculations.^37,38

We have chosen CDs in our work for two main reasons: 1) To assign the solution structures of the resulting CD complexes with a variety of structurally different azoalkanes based on ICD measurements, since the azo chromophore alignment inside the \( \beta \)-CD cavity has been recently established as a sensitive test for the orientation-intensity rules of ICD;^37 2) To use them as chiral discriminators to assess the thermodynamic and kinetic factors required for recognition of enantiomeric guests by means of spectroscopic techniques.

**Calixarenes.** Adolph von Bayer^39 pioneered the chemistry of calixarenes as products of the reaction of phenols with aldehydes in the presence of strong acids, but he was unable to determine its structure and did not realize its potential. In 1944, Zinker et al. proposed a cyclic tetrameric structure of the product of the base catalyzed condensation of \( p \)-tert-butylphenol and formaldehyde.^40 The first industrial application of calixarenes as demulsifiers started in the 1950s. Thereafter, the term of calixarene was coined in 1978 by D. Gutsche^41,42 and derives from "calix", the Greek word for cup and "arene", simply referring to the individual phenolic units that make up the cup.

Since Gutsche established the one-step synthetic route for facile synthesis of calixarenes from the condensation of \( p \)-alkylphenols and formaldehyde in good yields,^41-43
Calixarenes have rapidly gained research attention as macrocycles with (almost) unlimited possibilities in supramolecular chemistry because of their versatility and utility as host molecules after cyclodextrins and crown ethers.\textsuperscript{44-46} The development of this class of compounds relied on the availability of various ring sizes, and the ease of chemical modification. As illustrated in Scheme 1-3, the most common calixarene contains four, six or eight repetitive \textit{p}-\textit{tert}-butylphenolic units linked by methylene bridges forming a basket-shaped cavity which is characterized by a wider side containing the \textit{p}-\textit{tert}-butyl groups defined as the upper rim, and the narrower hydroxyl side as the lower rim.\textsuperscript{42} Cavity sizes of calixarenes bearing four, six and eight units are reported as 3.0, 7.6, and 11.7 Å, respectively.\textsuperscript{44} Although the parent calixarenes exist preferentially in the cone conformation, they are highly flexible and can adopt at least four up-down conformations (Scheme 1-4). The free rotations of the methylene groups between the phenols can bring about cone, partial cone, 1,2-alternate, and 1,3-alternate conformations.\textsuperscript{47} Their conformational flexibility can be controlled by functionalization of the upper and lower rims with proper bulky groups in order to decrease the rotational barrier.\textsuperscript{48,49} Among the many methods that can be used, NMR is one of the most useful to distinguish the four main conformations in the \textit{H} and \textit{C} NMR spectra,\textsuperscript{44,50} because the methylene bridges are strongly affected by conformational changes. Furthermore, X-ray crystallography provided conclusive proof for the viable conformations of calixarenes.

**Scheme 1-3. Parent calixarenes.**

\[
\text{Scheme 1-4. Schematic representation of calix}[4]\text{arene conformations.}
\]

The characteristic feature of calixarenes is their low insolubility in water, due to their dominant aromatic character. Considerable effort has been devoted in recent years to synthesize water-soluble derivatives. The first example was introduced by Ungaro\textsuperscript{51}, who
reported the functionalization of the lower rim of the parent calix[4]arene by carboxylate groups. Later, Shinkai reported the preparation of sulfonated calixarenes.\textsuperscript{52,53} Since then the pioneering work of Shinkai, other ionic derivatives from functionalization of the upper rim have emerged with different anionic and cationic moieties (Scheme 1-5).\textsuperscript{54-58}

**Scheme 1-5. Some examples of water-soluble calixarenes.**

![Diagram of calixarenes](image)

Among the water-soluble derivatives, the \( p \)-sulfonatocalixarenes have been the most widely studied because they possess the highest known aqueous solubility, > 0.1 M, and their synthesis is straightforward.\textsuperscript{52,53} The synthetic procedure of \( p \)-sulfonatocalix\([n]\)arenes (\( n = 4, 6, 8 \)) is shown in Scheme 1-6.

**Scheme 1-6. Synthesis of \( p \)-sulfonatocalix\([n]\)arenes (\( n = 4, 6, 8 \)).**

![Diagram of synthesis](image)

The sulfonatocalixarenes are often compared with CDs due to structural similarities (Table 1-1). Calixarenes provide not only a hydrophobic environment (benzene rings), but also hydrophilic head groups (\( \text{SO}_3^- \)), i.e., they possess properties of cyclodextrins and micelles. Their cavity is as hydrophobic as that of CDs. In contrast to CDs, their symmetrical structures render them achiral. The chirality can be induced by the introduction of chiral substituents\textsuperscript{59,60} or functional groups on the lower rim as well as by conformational isomerism.\textsuperscript{46} Chiral calixarenes are effective stationary phase for analytical separation techniques. Furthermore, they present some distinct spectral properties which have been
investigated using infrared (IR), UV, NMR, mass spectroscopy, and fluorescence spectroscopy. For example, the absorption maxima near 280 nm in the UV spectra can be red-shifted as well as the corresponding fluorescence emission spectra as a function of pH. The $pK_a$ (acid dissociation constant) for the OH groups have been determined by potentiometric $^{61,62}$ and photometric titrations. $^{63}$ Surprisingly, four very different $pK_a$ values were detected for nitro and sulfonated water-soluble calix[4]arenes. For example for $p$-sulfonatocalix[4]arene, the first dissociation ($pK_{a1}$ 3.26) occurs at acidic pH, whereas the other three dissociations ($pK_{a2}$ 11.80, $pK_{a3}$ 12.8, and $pK_{a4}$ ca. 14) are observed at relatively high pH. This has been related to a very strong intramolecular hydrogen bonding between the lower-rim hydroxyl groups.

Table 1-1. Comparison of properties of sulfonatocalixarenes and cyclodextrins.

<table>
<thead>
<tr>
<th>Property</th>
<th>Sulfonatocalix[n]arenes</th>
<th>Cyclodextrins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer</td>
<td>$p$-Sulfonatophenol</td>
<td>$\alpha$-D-Glucopyranose</td>
</tr>
<tr>
<td>Inner cavity diameter (Å)</td>
<td>3.0 (n = 4)</td>
<td>5.7 ($\alpha$)</td>
</tr>
<tr>
<td></td>
<td>7.6 (n = 6)</td>
<td>7.8 (β)</td>
</tr>
<tr>
<td></td>
<td>11.7 (n = 8)</td>
<td>9.5 (γ)</td>
</tr>
<tr>
<td>Cavity properties</td>
<td>Flexible</td>
<td>Rigid</td>
</tr>
<tr>
<td></td>
<td>Heterogeneous</td>
<td>Homogeneous</td>
</tr>
<tr>
<td>Solubility</td>
<td>Water soluble</td>
<td>Water soluble</td>
</tr>
<tr>
<td>Spectroscopic properties</td>
<td>Strong UV absorption</td>
<td>UV transparent</td>
</tr>
<tr>
<td>Interactions</td>
<td>Hydrophobic + electrostatic</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>Selective derivatization</td>
<td>Easy</td>
<td>Difficult</td>
</tr>
<tr>
<td>Inherent chirality</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

The interest in sulfonatocalixarenes for host-guest chemistry is largely due to their tendency to form complexes with a wide range of metal ions, noncharged and ionic organic molecules in aqueous media. In this context, their complexation behaviour towards metal cations, $^{64,65}$ fullerenes, $^{66,67}$ tetraalkylammonium, cholines, $^{68,69}$ and amino acids $^{70,72}$ have been documented. The contribution of hydrophobic and electrostatic (cation-$\pi$, $\pi$-$\pi$, and CH-$\pi$) interactions $^{73,74}$ has been reported to be important in the binding of guest molecules, and crucial for their molecular recognition properties. Guest size, shape complementarity, hydrophobicity and charge effects are key determinant in the selectivity of calixarenes, which has been extensively studied. $^{75}$ Results of theoretical and experimental investigations on calixarenes have contributed to the development of potential applications $^{75}$ in very diverse areas such as enzyme mimics, liquid-liquid metal extraction, selective ion transport, catalysis, $^{76}$ and sensors.$^{65,69,75}$ An environmentally relevant application of calixarenes is in nuclear waste treatment. A carboxylated derivative of $p$-sulfonatocalix[6]arene was
extensively used as a “super-uranophile” to selectively extract uranium from sea water through chromatographic separation methods.77,78 Others sulfonated and carboxylated calixarenes bearing crown ethers which exhibit high selectivity for strontium, cesium, and actinides derived from nuclear fission have been demonstrated as well.79,80

*p*-Sulfonatocalix[4]arene (CX4) is a second class of water-soluble supramolecular macrocycles with distinct properties. These studies were aimed at: 1) Demonstrating the importance of shape complementarity between host and guest molecules rather than hydrophobic effects in the molecular recognition process; 2) Evaluating its binding ability towards inorganic and organic cations through competitive binding assays by means of fluorescence-based method; and finally 3) Using CX4 as enzyme-mimetic building block to reproduce some enzymatic functions such as the alteration of chemical reactivity of bound substrates (large $pK_a$ shifts), and the structural specificity-dependence of metal-substrate interaction which is quintessential for biocatalysis in biochemical pathways.

### 1.3 Bicyclic Azoalkanes as Guests and Molecular Probes

![DBO, DBH, DBN, 3, 4, 5, 6](image)

During the last decades, azo-containing compounds have received considerable interest in photochemistry due to their propensity to undergo *cis-trans* isomerization upon light irradiation, and also due to the possibility to undergo nitrogen extrusion thermally or photochemically under varying conditions. The latter provides a clean and versatile source of radicals and biradicals.81 Among the most often investigated examples, the bicyclic *cis*-azoalkanes DBO, DBH, and DBN, which possess different ring sizes and a strained bicyclic skeleton, stand out. Their photolysis, and the product profile and distribution have been under extensive investigation. In most organic solvents, as well as inside host molecules, such as $\beta$-CD or zeolites, only two major products in varying proportions, 1,5-hexadiene (major) and bicycloc[2.2.0]hexane (minor), are formed during the photolysis of DBO, for example.

The common characteristics of azoalkanes is their $n,\pi^*$ azo chromophore which absorbs in the near-UV range. Their absorption spectra show a bathochromic shift with increasing ring size, from 331 nm for DBH to 396 nm for DBN. This series of aliphatic azoalkanes
exhibits a weak, but appreciable extinction coefficient depending on solvent polarity (50 M$^{-1}$cm$^{-1}$ in water and 200 M$^{-1}$cm$^{-1}$ in n-hexane for DBO). This is the result of a symmetry-allowed $n, \pi^*$ transition with a nonvanishing electric dipole transition moment directed along the $\pi$ orbitals of the azo $\pi$-system (arrow in Scheme 1-7).

**Scheme 1-7.** Electric dipole transition moment for the $n, \pi^*$ transition of cis-azoalkanes. The direction can be readily determined by multiplication of the relevant signs of the lobes of the pertinent $n$- and $\pi^*$ orbitals.

The high water solubility, structural diversity, and spectroscopic characteristics of azoalkanes render them suitable as guest molecules to investigate host-guest systems in aqueous solution. Host-guest complexation remains an excellent model for enzyme-substrate interactions. On the applied side, the azoalkane 2,3-diazobicyclo[2.2.2]oct-2-ene (DBO) has proven to be a useful molecular probe due to its solvatochromic behaviour, which can readily sense microenvironmental changes, e.g., the polarizability inside molecular containers. As additional advantage, the assignment of the direction of the electric dipole transition moment of the $n, \pi^*$ transition, relative to the axis of CDs as hosts, is helpful to examine the structure of CD complexes by the ICD method. The correlation between conformations of guest molecules inside the CD cavity and the ICD sign and intensity can be derived by using Harata’s and Kodaka’s rules. Such solution structures of host-guest complexes are intimately related to molecular recognition phenomena. Additionally, the specific location of functional groups inside the CD cavity can provide key information for relevant enzyme specificity.

An unusual feature is the noted high fluorescence quantum yield (ca. 20% in H$_2$O) observed for DBO, because most acyclic and cyclic azoalkanes don’t fluoresce at all. Among the bicyclic homologues, DBO possesses sufficient rigidity, but not too much ring strain and this combination allows this rare fluorescence phenomenon. Because of a strained [2.2.1] bicyclic skeleton, DBH undergoes a rapid nitrogen extrusion upon irradiation, whereas DBN, the more flexible one with a [2.2.3] skeleton, shows fast radiationless deactivation. The fluorescence spectrum of the singlet-excited state of DBO is broad ($\lambda_{\text{max}} \approx 430$ nm) and nearly unaffected by solvent or bridgehead substitution. The absorption and fluorescence spectra show only small solvatochromic shifts (Figure 1-1).

The most relevant and intriguing property of DBO is its extremely long fluorescence lifetime, which can reach 505 ns in aerated D$_2$O and 1 µs in gas phase, which is much longer
than pyrene (ca. 400 ns). However, the fluorescence of DBO is environmentally sensitive. Quenching can be induced by good hydrogen donors, such as antioxidants\textsuperscript{85,86} as well as some solvents,\textsuperscript{87,88} or with electron donors, such as amines.\textsuperscript{89} Interestingly, the quenching behaviour of its exited state is very useful in the design of chemical sensors.\textsuperscript{65,69}

**Figure 1-1. UV absorption and fluorescence spectra of DBO in D\textsubscript{2}O.**

Such a long-lived fluorescent probe with high photostability and sensitivity towards the microenvironment fulfils the requirements for the study of fast biomolecular and supramolecular kinetic processes in the nanosecond time range, which is relevant for most chemical reactions. In related approaches, photophysical probes (triplet-excited),\textsuperscript{90,91} or radicals\textsuperscript{92,93} have been applied for the measurement of fast rate constants of supramolecular association with CDs, which are in general on the order of $10^7$-$10^8$ M\textsuperscript{-1}s\textsuperscript{-1}. However, such probes present some drawbacks, because their detection by transient absorption (triplet states) or EPR (radicals) lacks sensitivity and time-resolution, respectively. In comparison, the advantages of fluorescent probes for sensing supramolecular events are very well-defined, including high sensitivity of detection down to the single molecule, excellent spatial and temporal resolution, and ease of detection.\textsuperscript{11} In this context, the photophysical properties of DBO have been investigated in detail and the synthetic possibility to readily tailor DBO derivatives led to the development of a series of fluorescent probes, which were referred to as Fluorazophores (“fluorescent azo chromophores”).\textsuperscript{94}

Recently, the members of the fluorazophore family have been employed as novel photophysical probes for direct determination of kinetic constants of host-guest complexation processes with cyclodextrins by means of time-resolved and steady-state fluorescence techniques.\textsuperscript{28,29} The fast quenching of their singlet-excited states induced by hydrogen abstraction from the inner glucose C-H bonds allows one to extract accurately the relevant entry and exit rate constants of the complexed fluorazophore, and respective lifetimes by analyzing the time-resolved fluorescence decay traces as well as the decrease of fluorescence
intensity. However, the extraction of the pertinent kinetic information from these experiments requires the knowledge of the precise quenching mechanisms. The binary systems, depicted in Scheme 1-8, are the simplest and have been predominantly studied.\textsuperscript{28,29} Regarding a 2:1 host-guest complexation, the kinetic scenario has not yet been realized. One tentative example of such a kinetic study has been proposed with the bifunctional probe 4, referred to as Fluorazophore-C\textsuperscript{95} (Scheme 1-9).

**Scheme 1-8. Binary quenching kinetics (1 indicates singlet-excited state).**

| \(k_{\text{ass}}\) | association rate constant for excited state |
| \(K\) | binding constant in ground state |
| \(k_{\text{diss}}\) | dissociation rate constant for excited-state complex |
| \(k_0\) | deactivation rate constant for excited state |
| \(k_{\text{CD}}\) | deactivation rate constant for excited-state complex |

**Scheme 1-9. Synthesis of Fluorazophore-C.**

The general synthetic procedure of DBO includes a Diels-Alder cycloaddition of a 1,3-cyclohexadiene and methyltriazolinedione used as azo dienophile.\textsuperscript{96,97} Subsequent catalytic hydrogenation of the double bond and hydrolysis by treatment with KOH in refluxing \(i\)-propanol yield the desired azoalkane. This method is suitable for most bridgehead-substituted fluorazophores,\textsuperscript{98} only if the appropriate 1,3-cyclohexadienes are
available. The hydroxymethyl-DBO, Fluorazophore-H, was used as a key compound as shown in Scheme 1-9 for the synthesis of Fluorazophore-C for chiral discrimination purpose.99

1.4 Scope of this Thesis

Their attractive structural characteristics, i.e., small size, spherical shape, different ring size, high solubility in water, their environmental responsiveness (photophysical properties of the azo chromophore) along with the long-lived fluorescence of the azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) render the class of bicyclic azoalkanes ideally as “smart probe molecules” in the field of supramolecular chemistry. Pioneered by our group, they offer quite remarkable insights into structural and functional aspects, allowing a better understanding of host-guest supramolecular assemblies, and thereby the development of supramolecular functional materials in general.

The aim of this thesis is to assess their versatility and applicability as guests and molecular probes by using novel analogues and derivatives of DBO in host-guest complexations. The research described herein deals with the study of the structure, thermodynamics and kinetics of their complexation with CDs and CX4, and the development of potential applications of the resulting novel properties of such supramolecular functional systems.

In the section concerning CDs, the azoalkanes are applied: 1) To elucidate solution structures of CD inclusion complexes. Six azoalkanes (DBO, DBH, DBN and 1-3) were selected as guest molecules to assign the structure of their resulting CD complexes by the ICD method, since DBO and the dialkylated derivative 5 were previously reported as typical examples, in which the azo chromophore alignment in chiral β-CD provided a sensitive tool for the orientation-intensity rules of ICD. In this part, the ICD rules are further tested in order to study the dependence of the conformations of the guests inside CDs, regarding the effect of the size of the guest and the host, as well as the influence of an additional chromophore; 2) Chiral discrimination through formation of diastereomeric host-guest complexes. The chiral fluorescent probe 4, composed of a camphanate moiety and DBO, was employed as model of chiral guests for this topic. The chiral discrimination with native β-CD was quantitatively analyzed through precipitation approach by means of NMR spectroscopy and circular dichroism, while the high solubility enhanced by methylated β-CD allowed us to perform this study using directly spectroscopic methods. The photophysical probe, DBO, was useful to determine the stoichiometry of the complexes and their binding constants, and also to assess the association rate constants and respective lifetimes for each enantiomers by means of ICD, UV and fluorescence titrations.
The section dealing with CX4, which was the most comprehensively investigated in this thesis, is subdivided into three topics: 1) Molecular recognition as well as solution structures studies of azoalkanes DBO, DBH, DBN, 3 and 5 with CX4 by using $^1$H and 2D NMR techniques. This study was designed to demonstrate the high spherical shape affinity of the CX4 conical cavity; 2) Supramolecular sensor systems. The CX4 inclusion complex of DBO allowed us to develop a fluorescent sensor to target organic and inorganic cations by means of displacement assays. Sensitivity of this system was further improved by using the derivative 6 which constitutes a promising biosensor for the development of highly demanding enzyme assays, and 3) Biomimetic supramolecular models. The first example is illustrated by the enzyme-like properties of CX4, which is able to alter the chemical reactivity of bound guests. This was exemplified by the large $pK_a$ shift of DBO, DBN and 5 upon inclusion. As a second example, the highly structural specificity-dependence of metal-substrate binding interaction, which occurred in the catalytic site of zinc-containing enzymes, was reproduced by supramolecular self-assembly by employing DBO and the azoalkanes 5 and 6 as substrates (S). The formation of stable CX4•S•Zn$^{2+}$ ternary complexes was monitored and quantified spectroscopically.
Chapter 2

SOLUTION STRUCTURES OF INCLUSION COMPLEXES
2.1 Induced Circular Dichroism and Structural Assignments of Cyclodextrin Complexes of Bicyclic Azoalkanes


Induced circular dichroism (ICD) is a powerful technique for structure analysis of cyclodextrin (CD) inclusion complexes, in particular the relative conformation of the guest to the host. The latter has been referred to as the “co-conformation” of the host-guest complex. The structural assignments of such complexes are based on two general ICD rules developed for 1:1 complexes by Harata and Kodaka. According to Harata’s rule, a positive ICD signal arises when the electric dipole transition moment of the chromophore inside the host cavity is aligned parallel to the axis of the host cavity, whereas a perpendicular orientation gives rise to a negative and half as strong signal (arrows in Scheme 2-1). A similar rule developed by Kodaka is applicable for guest positioned outside the host cavity.

Scheme 2-1. Harata’s and Kodaka’s rules.

The Harata’s rule has been recently investigated for aliphatic azoalkanes. The sign and intensity of the ICD signal is closely related to the relative orientation of the electric dipole transition moment of the azo chromophore, which points along the azo π orbital, with respect to the axis of CD (cf. Introduction, Section 1.3). In this present work, we selected the monochromophoric and bichromophoric azoalkanes 1-6 as guest molecules (Scheme 2-2) and different sizes of CDs such as α-CD (5 Å), β-CD (6 Å), and γ-CD (8 Å) (cf. Introduction, Section 1.2) to investigate by the ICD method the effect of the ring size and substitution.

Scheme 2-2. Monochromophoric and bichromophoric azoalkanes.
pattern of azoalkanes as well as of the type of host on the co-conformations of the resulting complexes.

**Monochromophoric azoalkanes.** The azoalkanes 1-4 with different ring sizes showed different ICD spectra. In β-CD, positive ICD effects were observed for 1, 2 and 4. The ICD intensity order (1 > 2 > 4) can be understood as a best size matching for 1 (5 Å diameter). Based on previous investigations for the β-CD complex of 1,\(^{37}\) in which a preferential lateral co-conformation was demonstrated, similar co-conformations were suggested, in which the electric dipole transition moment is oriented along the CD axis (arrow in Scheme 2-3). Regarding the negative ICD for the 7,7-dimethyl derivative 3 with β-CD, two alternative interpretations were proposed: 1) A deep immersion of the dimethylmethylene bridge, pushing the azo group outside the cavity, could account for the predicted opposite ICD according to Harata’s rule; 2) Steric effects may cause a tilted lateral alignment which could invert the ICD sign. In γ-CD, the ICD effects of 1-4 were all weak and positive. Again, the preference for a lateral co-conformation was proposed as well.

**Scheme 2-3. Co-conformation of β-CD•1 complex.**

![Scheme 2-3. Co-conformation of β-CD•1 complex.](image)

The emerging fine structure of 3 with α-CD was interpreted in terms of the formation of a tight 2:1 host•guest complex. In this case, no structural assignment of the ICD sign for the 2:1 complex could be reliably derived from Harata’s and Kodada’s rules. Our molecular mechanics simulation confirmed a similar complex structure as that suggested on the basis of NMR for the structurally related camphor for its corresponding 2:1 complex.\(^{102}\)

**Bichromophoric azoalkanes.** In β-CD, azoalkane 5 shows a weak positive band at 375 nm (azo chromophore) and stronger below 270 nm (aromatic moiety) (Figure 2-1a). Because of a deep inclusion of the most hydrophobic aromatic residue, as confirmed by strong cross-peaks between the aromatic protons and the inner CD protons in 2D REOSY NMR spectra (Figure 2-1b), an axial co-conformation with the DBO moiety located outside β-CD was found (Scheme 2-4). Also supported by the computationally optimized complex structure depicted in Scheme 2-4, the structural assignment of the positive ICD band for the azo group was in good agreement with Kodaka’s rule, since the electric dipole transition moment lies outside and is oriented perpendicular to the cavity axis. Similar co-conformations were also involved to explain the positive ICD signals with α- and γ-CDs, since the molecular length of 5 (ca. 10 Å) exceeds the depth of CDs (8 Å).
Chapter 2

Solution Structures of Inclusion Complexes

Figure 2-1. (a) ICD spectra of 0.5 mM azoalkane 5 in the presence of 12 mM β-CD in D₂O; (b) Diagnostic region of the 2D ROESY NMR spectrum of the β-CD•5 complex in D₂O.

Scheme 2-4. Co-conformation of β-CD•6 complex.

However, in contrast to 5, the ICD spectrum of the bisazoalkane 6 with β-CD displayed a stronger positive band at 370 nm and a weaker negative one around 385 nm. According to the peculiar ICD titration behaviour, the respective bands were consequently assigned to the ICD signal of the 1:1 complex, and to that of the 2:1 complex, which emerged at high β-CD concentrations. As noted for 3, a definitive assignment on the basis of the classical ICD rules was not possible, due to the presence of two identical azo chromophores.

In summary, the solution structures of the complexes can be assigned on the basis of Harata’s and Kodaka’s rules in some limiting cases. In the majority of cases, the azoalkanes adapt preferentially a deeply immersed lateral co-conformation within the cavity of CDs, in line with better hydrophobic interactions and size matching between host and guest.
2.2 Spherical Shape Complementarity as an Overriding Motif in the Molecular Recognition of Noncharged Guests by p-Sulfonatocalix[4]arene: Complexation of Bicyclic Azoalkanes


In this present work, we focused our investigations on the binding constants and inclusion geometries of host-guest complexes on the basis of the strong shape complementarity, thereby optimizing CH-π interactions between p-sulfonatocalix[4]arene (CX4) and azoalkanes 1-5 in neutral aqueous solution. The exceptional shape affinity of CX4 is reflected in substantially higher binding constants (up to 1000 M\(^{-1}\)) of 1-3 compared to those reported for noncharged organic guest molecules (10-100 M\(^{-1}\)).\(^{2,107-109}\) This suggested that the CX4 cavity (6.0-6.3 Å) provided an ideal spherical space to match guest volumes of 110-130 Å\(^3\) (Figure 2-2a).

**Scheme 2-5.** Proton labelling for guests, and suggested structures for the CX4 inclusion complexes; Regions with significant 2D ROESY crosspeaks are marked in green and blue.

Because of its conformational flexibility, the CX4 pentanion can also adapt the inner conical cavity size through an induced fit. This resulted in a moderate guest size selectivity as
shown in Figure 2-2a. In this context, (+)-camphor was selected as a reference bicyclic and spherical guest to generalize the importance of the spherical size complementarity.

**Figure 2-2.** (a) Apparent relationship between the binding constants of bicyclic guests with CX4 and the calculated volume of the guest; (b) Diagnostic region of the 2D ROESY spectrum for the CX4 inclusion complex of azoalkane 5 in D2O.

Larger complexation-induced upfield shifts for the exo protons, and 2D ROESY crosspeaks observed for the bridgehead protons H_b suggested an equatorial inclusion complex for azoalkanes 1-3 (Scheme 2-5), where the polar azo group points towards the aqueous bulk and where the hydrophobic part of the bicycle can efficiently interact with CX4 through CH-π interactions. For azoalkanes 4 and 5, an axial inclusion geometry (Scheme 2-5) was supported by the deep immersion of the isopropyl group of 4 (largest shifts for the isopropyl group), and by the position of the phenyl ring of 5 towards the aqueous bulk (very small shifts for the phenyl protons, and significant crosspeaks for exo and endo’ protons of the bicycle, Figure 2-2b). Accordingly, the inclusion pattern of 5 was exactly opposite to that observed with β-CD (cf. Section 2.1).

Most importantly, these results demonstrate the spherical shape complementarity as an important factor in the molecular recognition process, which overrides hydrophobic and π-π interactions in the complexation with CX4. This consideration can be taken into account as a design criterion to optimize the binding of noncharged organic guests with p-sulfonatocalixarenes.
Chapter 3

CHIRAL DISCRIMINATION THROUGH HOST-GUEST COMPLEXATION
3.1 Chiral Resolution Through Precipitation of Diastereomeric Capsules in the Form of 2:1 β-Cyclodextrin-Guest Complexes


The general concept, which is based on the host-induced precipitation of the guest, relies on the formation of precipitating diastereomeric capsules self-assembled from chiral guest molecules and two chiral hosts in water (Scheme 3-1). The presently explored method allows for a chiral resolution of racemate solutions on account of differential binding constants as well as solubility of the 2:1 complexes. The enantiomeric enrichment in the precipitate can be easily and accurately determined by the ICD method.

Scheme 3-1. Chiral resolution through self-assembled encapsulation.

For this approach, we have selected 1, the (+)/(−)-enantiomeric camphanate esters of DBO, as chiral guest molecules and the natural β-CD as chiral selector. For reference purposes, we also prepared the individual components, i.e., the enantiopure ethyl camphanates 2 as well as the achiral acetate 3.

The characterization of the precipitate as a 2:1 complex by 1H-NMR allowed us to perform a quantitative analysis of the intricate complexation equilibrium involving the formation of 1:1 and 2:1 CD complexes with each enantiomer of 1 displaying differential precipitation of the 2:1 complex. The differential binding constants for 1:1 as well as 2:1 complex formation resulted in a lower solubility of the (−)-enantiomer in the presence of β-CD. In control experiments, no change in the solubility of reference compounds 2 and 3 was observed, suggesting that they only form soluble 1:1 complexes.

To test practical usefulness of this method as a novel route for chiral resolution, a precipitation from a racemic solution of 1 by addition of β-CD was performed. The circular dichroism spectra of the redissolved precipitate and the supernatant solution immediately
indicated that the solution had been enriched with the (+)-enantiomer, and the precipitate with the (−)-form (Figure 3-1a), in line with the projected solubility results. The enantiomeric excess ($ee$) in the precipitate was determined as $30 \pm 3\%$ from calibration lines (Figure 3-1b).

**Figure 3-1.** (a) *Circular dichroism analysis of the precipitation process induced by adding 5 mM β-CD to a 2 mM racemic solution of I; Spectra of the supernatant solution after precipitation (---) and the solution of redissolved precipitate (−) in D$_2$O; (b) Determination of the enantiomeric excess ($ee$) in the precipitate from calibration lines.*

While moderate, this value is actually comparable to the optical purity achieved in preparative crystal growth experiments with modified CDs,$^{103,104}$ and larger than the $ee$ achieved in the original precipitation experiments by Cramer and Dietsche,$^{105}$ which were conducted in the presence of a large excess of guest. This establishes the present method as a rational approach to chiral resolution, although the absolute variation of the binding constants to which the enantiomeric excess is closely related (equation in Figure 3-1b), and therefore the chiral resolution is relatively small for the presently selected pair of enantiomers. Other pairs of enantiomers could be tested in order to improve the preliminary results.
3.2 Chiral Discrimination in the Complexation of Heptakis-(2,6-di-O-methyl)-β-cyclodextrin with 2,3-Diazabicyclo[2.2.2]oct-2-ene Derivatives


In this work, the chiral discrimination of enantiomeric guests 1 by the derivative heptakis-(2,6-di-O-methyl)-β-cyclodextrin (DMe-β-CD) was studied. Instead of natural β-CD, DMe-β-CD, in which the 2-OH and 6-OH groups are methylated (see structure below), was employed because of its higher water-solubility, which suppressed the precipitation of its complexes and allowed additionally the use of higher host concentrations to study more reliably the sequential formation of the 1:1 and 2:1 complexes directly spectroscopically. ICD, UV spectrophotometry, and fluorescence spectroscopy were used in order to establish relationships between structural, thermodynamic and kinetic aspects of the chirality-based molecular recognition process.

Complexation-induced ICD and UV spectral changes of the photophysical probe (DBO) were investigated to clearly understand the complexation behaviour of 1 with DMe-β-CD as well as to determine the ground-state binding constants $K_1$ and $K_2$ of the ternary complexes, which are required for kinetic analysis. The competitive inclusion of the camphane moiety was deduced from the inversion of the sign of the ICD band of the n,π* transition of the azo chromophore (Scheme 3-2). For the (+)-enantiomer, the negative effect was assigned to the ICD induced in the 1:1 complex, and the positive effect to that induced in the 2:1 complex (Figure 3-2a). The ICD behaviour of the (−)-enantiomer was qualitatively opposite (Figure 3-2b). Also supported by the UV-spectral behaviour, the well-defined isosbestic point at about 367 nm, which accounts for the initial formation of a 1:1 complex, was followed by a second one at around 373 characterizing the inclusion of DBO residue in the second complex, which was in line with an increase of its extinction coefficient in response to the microenvironmental change inside the CD cavity.
Scheme 3-2. Inversion of ICD sign for the formation of 1:1 and 2:1 host-guest inclusion complexes.

Compared to achiral azoalkanes, the rules of Harata and Kodaka could not be applied to predict the co-conformations of the inclusion complexes with chiral guests. The opposite ICD for both enantiomers is therefore unlikely to be related to axial co-conformations, as would be required by the above rules. However, Kodaka's rule predicts an inversion of the sign of the ICD when the chromophore is positioned in the same relative orientation (with respect to the CD axis) inside or outside the CD cavity. It is therefore reasonable to expect different signs when the azo chromophore is positioned outside the CD cavity (1:1 complex) or inside the CD (2:1 complex), such that the inversion of the ICD upon forming the 2:1 complex (Figure 3-2) can be nicely rationalized for both enantiomers.

Figure 3-2. Induced circular dichroism spectra of (a) (+)-I and (b) (−)-I (2 mM) in D₂O at various concentrations of DMe-β-CD, and (c) corresponding titration plots ($\theta_{obs} = 370$ nm).

The pertinent binding constants $K_1$ and $K_2$ (Scheme 3-3, bottom) were derived, from plots of the maximum spectral changes (UV absorbance or ellipticity) versus host concentration, by means of a cubic equation-based fitting procedure (Figure 3-2c). Both methods afforded a ca. 50 times higher binding constants for formation of the 1:1 complex ($K_1$) than for the 2:1 complex ($K_2$). Although the absolute values of the two independent titration methods varied slightly, both suggested a 20% higher $K_1$ value for complexation of the (+)-enantiomer. The binding constants for the $K_2$ value showed the opposite trend (larger value for (−)-enantiomer).
The quantitative analysis of the steady-state fluorescence quenching experiments allowed us to extract the quintessential kinetic parameters of the excited-state complexation equilibria (Scheme 3-3, top) which are the association rate constants $k_{ass1}$ for the formation of the 1:1 complexes along with the fluorescence lifetimes of the 1:1 and 2:1 complexes $\tau_{CD1}$ and $\tau_{CD2}$ of both enantiomers. A statistically significant chiral discrimination of the kinetics of association as well as for the corresponding fluorescence lifetimes was found, regardless of whether the binding constants have been taken from either the ICD or UV absorption titrations. In fact, while close to the diffusion-controlled limit ($10^9 \text{ M}^{-1}\text{s}^{-1}$), the rate constants $k_{ass1}$ were measured as ca. 20% faster for the (–)-enantiomer, and consistently $\tau_{CD1}$ and $\tau_{CD2}$ were jointly shorter for the (+)-enantiomer.

**Scheme 3-3. Ternary quenching kinetics.**

These results revealed no direct correlation between the thermodynamics and kinetics data, since the stronger binding constant of (+)-enantiomer showed a slower rate constant for complexation. Similar observations were made in previous studies of CD complexation.\textsuperscript{29,106}
Chapter 4

SUPRAMOLECULAR FLUORESCENT SENSORS
4.1 Fluorescence Regeneration as Signaling Principle for Choline and Carnitine Binding by \( p \)-Sulfonatocalix[4]arene: A Refined Supramolecular Sensor System Based on a Fluorescent Azoalkane


As discussed previously in Section 2.2, DBO can form a deep 1:1 CX4 inclusion complex. Furthermore, the fluorescence emission of DBO was strongly quenched upon complexation, especially at low pH (2.4). The stronger quenching in acidic condition was rationalized in terms of stronger binding constant (4700 M\(^{-1}\)), which resulted in the induced protonation of DBO in the CX4•DBO complex (*cf.* Section 5.1). Combined with the well-established cation-receptor property of CX4,\(^{110,111}\) the potential of the resulting complex paved the way for applications as fluorescent sensor to signal the binding of organic ammonium ions over a large pH range.

Scheme 4-1. Sensor system based on the fluorescence regeneration of DBO.

The working principle of this sensor system relies on the efficient fluorescence regeneration of DBO from CX4 by competitive binding of analytes (Scheme 4-1), as follows: The addition of the organic cation displaces DBO (or protonated DBO, at low pH) in the complex, which is therefore released into the aqueous bulk, where static fluorescence quenching by CX4 no longer applies.

Owing to their biochemical importance and structural similarity, the derivatives of choline and carnitine (see structures below) were subject of our study. The fluorescence recovery of the uncomplexed DBO from the decomposition of the complex (1 mM DBO, 1.6 mM CX4) was monitored by steady-state fluorescence method at acidic and neutral pH (Figure 4-1a). The fitting of the fluorescence titration data according to a competitive binding model (Figure 4-1b) afforded higher binding constants for the cholines (ca. 10^5 M\(^{-1}\)) in neutral than in acidic solutions, which were in good agreement with literature values obtained.
by $^1$H-NMR. This reflected the enhanced stabilization of the alkyltrimethylammonium recognition motif by the upper-rim sulfonato groups through cation-π interactions.\cite{68,112-115}

<p>| | | |</p>
<table>
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<tbody>
<tr>
<td>choline</td>
<td>OH</td>
<td>H</td>
</tr>
<tr>
<td>acetylcholine</td>
<td>OCOCH$_3$</td>
<td>H</td>
</tr>
<tr>
<td>carbamoylcholine</td>
<td>OCONH$_2$</td>
<td>H</td>
</tr>
<tr>
<td>L-carnitine</td>
<td>CH$_2$CO$_2^-$</td>
<td>OH</td>
</tr>
<tr>
<td>O-acetyl-L-carnitine</td>
<td>CH$_2$CO$_2^-$</td>
<td>OCOCH$_3$</td>
</tr>
</tbody>
</table>

**Figure 4-1.** (a) Fluorescence regeneration upon successive addition of acetylcholine at pD 2.4. The inset shows the increase in relative fluorescence intensity of 1 mM DBO in presence of 1.6 mM CX4 for acetylcholine; (b) Increase in relative fluorescence intensity for choline and L-carnitine at pD 7.4, fitted according to a competitive binding model.

In contrast, the presence of a carboxylate in the carnitines resulted in a more than one order of magnitude weaker binding, as a consequence of the charge repulsion between the sulfonato and carboxylate groups (Figure 4-1b). Unlike the high selectivity for cholines, a relatively poor discrimination by CX4 was observed between acetylated, carbamylated and unsubstituted cholines, presumably due to the exposition of the noncharged substituted group (R in Scheme 4-1) to the aqueous bulk.


Due to the efficiency of our fluorescence-based method, as demonstrated for organic cations, the system was also applied to conveniently study the binding of inorganic cations with CX4 in D$_2$O at different pD (Scheme 4-1 in Section 4.1). The fluorescence release of DBO upon addition of monovalent (alkali), divalent (alkali earth), and trivalent cations to the same DBO/CX4 concentration as that used in Section 4.1 was considerably different. Indeed, while most metals like Na$^+$ or Mg$^{2+}$ displayed smaller effects (Figure 4-2a), the release was quantitative for some metals, e.g., for Al$^{3+}$ or La$^{3+}$ (Figure 4-2).

**Figure 4-2.** Increase in relative fluorescence intensity of 1 mM DBO in the presence of 1.6 mM CX4 illustrating the dependence (a) on cation charge at pD 2.4, and (b) on pD for La$^{3+}$.

![Figure 4-2](image)

The resulting data set in Table 4-1 revealed that the binding constants were dependent on the charge and the size of cations. In acidic solution, the weaker binding of alkali ions (on the order of 100 M$^{-1}$) than those for quaternary ammoniums ions suggested the importance of hydrophobic interactions for organic cations.$^{68,114}$ Regarding the size selectivity, the largest cesium gave rise to the highest binding constant, twice higher than the smallest lithium. Similar size selectivity trend was also observed for alkali earth cations. Al$^{3+}$, Ga$^{3+}$, and La$^{3+}$ displayed the largest binding constants, all close to 20000 M$^{-1}$. Similar values resulting from microcalorimetry titrations were reported as 2000 M$^{-1}$ for Mg$^{2+}$ and Ca$^{2+}$, and 17000 M$^{-1}$ for La$^{3+}$. The fluorescence regeneration and the competitive binding of the cations were also monitored at pD 7.4 (Figure 4-2b, Table 4-1). Taking Ca$^{2+}$ as representative metal ion, the possibility to follow the release by using other spectroscopic techniques confirmed the accuracy of our method (values in square brackets in Table 4-1). The larger binding constants in neutral solution were consistently accounted for increased cation-\( \pi \) interactions between the CX4 pentaanion and the cations.$^{114}$
Table 4-1. Ionic radii (Å) and binding constants$^a$ of different ions with CX4 in D$_2$O determined by fluorescence regeneration.

<table>
<thead>
<tr>
<th>ion</th>
<th>radius [Å]$^b$</th>
<th>$K$ [M$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pD 2.4</td>
<td>pD 7.4</td>
</tr>
<tr>
<td>NH$_4^+$</td>
<td>1.36</td>
<td>95</td>
</tr>
<tr>
<td>Li$^+$</td>
<td>0.59</td>
<td>70</td>
</tr>
<tr>
<td>Na$^+$</td>
<td>1.02</td>
<td>75</td>
</tr>
<tr>
<td>K$^+$</td>
<td>1.37</td>
<td>100</td>
</tr>
<tr>
<td>Rb$^+$</td>
<td>1.52</td>
<td>110</td>
</tr>
<tr>
<td>Cs$^+$</td>
<td>1.67</td>
<td>150</td>
</tr>
<tr>
<td>Mg$^{2+}$</td>
<td>0.57</td>
<td>1020</td>
</tr>
<tr>
<td>Ca$^{2+}$</td>
<td>1.00</td>
<td>1590 [1720]$^c$ [1640]$^d$</td>
</tr>
<tr>
<td>Sr$^{2+}$</td>
<td>1.18</td>
<td>1810</td>
</tr>
<tr>
<td>Ba$^{2+}$</td>
<td>1.35</td>
<td>$e$</td>
</tr>
<tr>
<td>Al$^{3+}$</td>
<td>0.39</td>
<td>20300</td>
</tr>
<tr>
<td>Ga$^{3+}$</td>
<td>0.47</td>
<td>25200</td>
</tr>
<tr>
<td>La$^{3+}$</td>
<td>1.03</td>
<td>23700</td>
</tr>
</tbody>
</table>

$^a$ 10 % error unless explicitly stated. $^b$ From Handbook of Chemistry and Physics, ed. D. R. Lide, Boca Raton, London, 84th ed., 2003. $^c$ By UV absorption titration. $^d$ By $^1$H NMR titration. $^e$ Precipitation above 1 mM.

The readily detectable binding of inorganic monovalent cations (K$^+$ and NH$_4^+$) by competitive fluorophore displacement provided strong circumstantial evidence for a purely entropically driven complexation process, which has been previously considered as a scarce possibility.$^{109}$ In most complexation studies with CX4, the adverse effects of buffer constituents, e.g., 0.1 M phosphate buffer,$^{68,107,108,114}$ both in acidic and neutral aqueous solution has been implicitly neglected. As demonstrated herein, this cannot be neglected, since the accuracy of reported binding constants depended critically on the actual metal ion concentrations present.
4.3 Label-Free Supra-Biomolecular Tandem Assays


In recent years, great efforts have been devoted to the development of enzyme assays for high-throughput screening.\textsuperscript{116,117} In this respect, new reaction types and new assay concepts have been reported. Most high-throughput assays used in drug discovery are based on chromogenic and fluorogenic substrates or color and fluorescent sensors\textsuperscript{118-120} in order to facilitate the spectroscopic detection of enzymatic transformations. As a promising system for this purpose, the previously developed sensor based on the $p$-sulfonatocalix[4]arene (CX4) and DBO was further refined.

First, the functionalization of the DBO fluorophore by an ammonium group (1) allowed remarkably enhanced binding constants ($1.2 \times 10^4$ M$^{-1}$ at pD 2.4 and $6 \times 10^4$ M$^{-1}$ at pD 7.4 in D$_2$O, and $4 \times 10^4$ M$^{-1}$ at pH 4.5 in H$_2$O) for the CX4•1 complex as well as much more stronger fluorescence quenching of 1. Second, the sensitivity of the sensor and the fluorescence release were much more appreciable, thereby allowing one to perform now displacement assays in the micromolar concentration range. For example, as shown in Figure 4-3, the quantitative fluorescence regeneration upon addition of acetylcholine to 0.25 mM 1 and 0.4 mM CX4 at pD 7.4 can be monitored, and the release is reproducible in an excellent manner by using either steady-state (Figure 4-3a) or time-resolved (Figure 4-3b) fluorescence techniques. Overall, both measurements first allowed one to reproduce the binding constant value for acetylcholine as that determined with the CX4•DBO system, and second displayed a more than 10 fold-fluorescence enhancement, which has greatly been improved by a factor of five in comparison to the DBO response. Keeping in mind that CX4 exhibits its unique selectivity only through electrostatic interactions, the improved sensor system, which offers now several practical advantages, can be projected to become a rapid and convenient method in the development of new enzyme assays. Here I present some preliminary results related to our label-free supra-biomolecular tandem assay. This project is now being pursued by Andreas Hennig from our research group.

Due to the quite outstanding experimental results obtained through displacement assays (inset in Figure 4-4b, Figure 4-5b), the newly designed biosensor, based on a simple inclusion
complex formation of 100 μM 1 with 200 μM CX4 was first applied in the study of the enzymatic conversion of L-arginine to L-ornithine (Figure 4-4a). An additional bioassay will be performed for the conversion of L-histidine to histamine via the action of L-histidine decarboxylase (Figure 4-5a). Both enzymatic reactions were chosen based on their important physiological implications.

Figure 4-3. Variation of the fluorescence (a) spectra and (b) decays of 0.25 mM 1 in the presence of 0.40 mM CX4 at pH 7.4 upon successive addition of acetylcholine. The insets show the increase in relative (a) fluorescence intensity and (b) the number of counts at the same 1/CX4 concentration, fitted according to a competitive binding model.

Figure 4-4. (a) Enzymatic conversion of L-arginine to L-ornithine by L-arginase; (b) Kinetic trace monitored by steady-state fluorescence at pH 9.5. The inset shows the increase in relative fluorescence intensity of 100 μM 1 in the presence of 200 μM CX4 in H2O upon successive addition of analytes at pH 9.5.

According to our independently performed competitive binding experiments (inset in Figure 4-4b), CX4 displayed higher binding affinity for L-arginine compared to L-ornithine. In fact, one anticipates a less favourable space-filling interaction in the binding of L-ornithine with CX4 (smaller ammonium group). Note also that no binding of urea by CX4 was observed. With respect to these results, the enzyme assay carried out by my colleague Andreas Hennig. The kinetic trace which was monitored by steady-state fluorescence upon
injection of L-arginase to a 1 mM L-arginine solution adjusted at pH 9.5 showed a decrease of the fluorescence intensity by the hydrolysis of the guanidinium group of L-arginine (Figure 4-4b. This provided a satisfactory reproduction of the projected data resulting from competitive binding measurements. The working principle of our tandem assay is illustrated in details in Scheme 4-2.

Scheme 4-2. Working principle of the tandem assay for L-arginase.

![Scheme 4-2](image)

Figure 4-5. (a) Enzymatic conversion of L-histidine to histamine by L-histidine decarboxylase; (b) Increase in relative fluorescence intensity of 100 µM 1 in the presence of 200 µM CX4 in H₂O upon successive addition of analytes at pH 4.5, fitted according to a competitive binding model.

However, in the case of the second biochemical reaction, the decarboxylation of the amino acid L-histidine should result in a quantitative fluorescence release. This is reflected in Figure 4-5b by the more favourable electrostatic contribution with the doubly positively charged histamine, as indicated by the higher binding constant (1.3x10⁵ M⁻¹ versus 0.5 x10⁵ M⁻¹ for L-histidine) at pH 4.5, which promises an improved performance for L-histidine decarboxylase.
Chapter 5

BIOMIMETIC SUPRAMOLECULAR MODELS
5.1 Analysis of Host-Assisted Guest Protonation Exemplified for p-Sulfonatocalix[4]arene – Towards Enzyme-Mimetic pK\textsubscript{a} Shifts


This work involves a system with an enzyme mimetic-supramolecular activity that possesses the characteristic properties of binding guest molecules by intermolecular interactions and then altering their chemical reactivity, e.g., basicity or acidity constant, quantum yield or potential redox. The essential requirement for a host molecule to possess enzyme-like activity are (i) a cavity complementarity in size and shape to that of the guest molecule in order to bind the guest and (ii) appropriately positioned functional groups to interact with specific sites of the guest molecule. The simple way for chemical transformations of bound guests by supramolecular inclusion has already opened up routes for catalytic and biomimetic applications.\textsuperscript{14,121}

Figure 5-1. (a) pD titration plots for the UV absorption (365 nm) of 2 mM 2 in D\textsubscript{2}O in the absence (filled circles) and presence (open circles) of 4 mM CX4; (b) Binding constants of the CX4•2 inclusion complex in dependence of pD in D\textsubscript{2}O.

Our present investigation documents a quantitative analysis of an interesting case of host-assisted guest protonation revealed for CX4, thereby accounting for an increased basicity accompanied by a large pK\textsubscript{a} shift of the guests 2-4 by about 2 units. Related pK\textsubscript{a} shifts resulting from hydrophobic and electrostatic effects are abundantly well documented in many biological systems, where protein-assisted protonation or deprotonation of substrates is of utmost importance for enzymatic activity.\textsuperscript{122-125}
Bicyclic azoalkanes are very weak bases (pK\textsubscript{a} values < 1.5 in D\textsubscript{2}O, Table 5-1). Their sizable protonation within the CX4 complex was directly indicated by the decrease of the near-UV absorbance. Because of its lowest pK\textsubscript{a} value (Table 5-1), the azoalkane 1 remained unprotonated. The decrease of the near-UV absorbance of 2-4 (2 mM) in the absence and presence of 4 mM CX4 was therefore spectrophotometrically monitored at varying pD (Figure 5-1a). The fitting of the titration data in the presence of CX4 according to a four-state complexation model (Scheme 5-1), considering absorbance contributions from four different forms of 2 (the complexed and uncomplexed, and protonated and unprotonated forms) afforded a pK\textsubscript{a} value of 2.5 for the CX4•2 complex. Similar UV spectrophotometric pD titrations were performed for the azoalkanes 3 and 4 and afforded pK\textsubscript{a} shifts of 2 units (pK\textsubscript{a}'(UV) in Table 5-1).

**Table 5-1.** pK\textsubscript{a} values of azoalkanes 1-4 in their uncomplexed (pK\textsubscript{a}) and CX4 complexed state (pK\textsubscript{a}') in D\textsubscript{2}O and extrapolated binding constants of the unprotonated and protonated azoalkanes 1-4.

<table>
<thead>
<tr>
<th>azoalkane</th>
<th>pK\textsubscript{a}</th>
<th>pK\textsubscript{a}' (UV)</th>
<th>pK\textsubscript{a}' (NMR)</th>
<th>ΔpK\textsubscript{a}</th>
<th>K\textsubscript{G,1} [10^3 M\textsuperscript{-1}]\textsuperscript{a,c} (unprotonated)</th>
<th>K\textsubscript{GH+} [10^5 M\textsuperscript{-1}]\textsuperscript{b,c} (protonated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>[−0.8]\textsuperscript{d}</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>[0.69]\textsuperscript{i}</td>
<td>---</td>
</tr>
<tr>
<td>2</td>
<td>1.0\textsuperscript{f}</td>
<td>2.5</td>
<td>3.0</td>
<td>1.8 ± 0.3</td>
<td>1.0\textsuperscript{f}</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>1.5\textsuperscript{g}</td>
<td>3.4</td>
<td>3.9</td>
<td>2.2 ± 0.3</td>
<td>[0.95]\textsuperscript{i}</td>
<td>2.5</td>
</tr>
<tr>
<td>4</td>
<td>1.1\textsuperscript{h}</td>
<td>2.6</td>
<td>3.1</td>
<td>1.8 ± 0.3</td>
<td>[0.48]\textsuperscript{i}</td>
<td>0.49</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Binding constant of the unprotonated azoalkane with the CX4 tetraanion, cf. Scheme 5-1. \textsuperscript{b} Binding constant of the protonated azoalkane with the CX4 tetraanion, cf. Scheme 5-1. \textsuperscript{c} Obtained by fitting the experimental binding constants below pD 8 according to a seven-state model, cf. Scheme 5-1. \textsuperscript{d} Estimated from the value of −1.4 in H\textsubscript{2}O from ref.\textsuperscript{126} by adding 0.6 units as a typical offset for D\textsubscript{2}O, cf. ref.\textsuperscript{127,128}. \textsuperscript{e} Taken as the binding constant at pD 7.4, cf. Section 4. \textsuperscript{f} This work; the value for 2 in H\textsubscript{2}O was determined as 0.5, which compares with a value of 0.4 reported in ref.\textsuperscript{126}. \textsuperscript{g} This work; the 3 value of 3.0 from ref.\textsuperscript{126} in H\textsubscript{2}O appears to be in error. \textsuperscript{h} This work.

Furthermore, the analysis of the pD-dependence of the binding constants for the CX4 complexes of azoalkanes 2-4 derived independently from \textsuperscript{1}H-NMR experiments, and fitted according to a seven-state model (Figure 5-1b), produced data, which compared very well the pK\textsubscript{a} shifts obtained from UV spectrophotometric titrations (pK\textsubscript{a}'(NMR) in Table 5-1). The slight difference in pK\textsubscript{a} shifts can be presumably related to the restriction of our four-state protonation equilibrium, already complicated at this level. The decrease in binding constants below pD 1 observed in Figure 5-1b was essentially due to the competition of the deuterated hydronium ion (Scheme 5-1). Owing to pK\textsubscript{a} shifts by 2 units, stronger binding constants (ca. 10^5 M\textsuperscript{-1}) can be extrapolated for the entirely complexed protonated guests. Regarding their complex structures, while the postulated equatorial conformation for the azoalkanes 2 and 3 in neutral solution remained unchanged in acidic condition (pD 2.4), the larger shift of the
bridgehead methyl group of 4 relative to that of the isopropyl group suggested a tilted axial complex geometry (Scheme 5-2).

**Scheme 5-1.** Mechanism for the complexation of azoalkanes with CX4 in D₂O (exchanged deuterium atomes are not shown for clarity).

As can be seen in Table 5-1, the protonation of noncharged guest molecules can increase the binding affinity by a factor of 100, as a consequence of the strong electrostatic contribution. This observed enhancement upon protonation compares very well with the acid-catalyzed methanolysis of N-acetyl-L-amino acids, which can be accelerated from 10 to 86 times in presence of CX4.¹²⁹

**Scheme 5-2.** Presumed complexation geometries for the CX4 complexes (tetraanion, acidic pD) of the protonated azoalkanes 2-4.
The case of host-assisted guest protonation appears to be a rather general phenomenon for macrocyclic cation acceptors, as demonstrated previously for cucurbit[6]uril.\textsuperscript{130,131} The \(pK_a\) shifts in the calixarene are larger due to stronger electrostatic interactions (ion-ion \textit{versus} ion-dipole). The design and understanding of supramolecular systems mimicking this enzymatic action presents therefore a great challenge, which should provide an incentive to study additional cation and anion receptors with respect to their ability to modulate the protonation equilibria of organic guests. In contrast to enzymes, CX4 shows, however, a rather low selectivity in binding and protonating guest molecules. This can be presumably related to the flexible nature of CX4, which can partially adjust its geometry to optimize both hydrophobic and electrostatic interactions. Future research will therefore be directed towards host molecules, which display more selective binding, more selective \(pK_a\) shifts, or both.
Chapter 5

5.2 Calixarene-Based Zinc Metallo-Enzyme Models Exhibiting Structural Specificity-Dependence of Metal-Substrate Binding Interaction


Zinc is the second most abundant transition metal in the body. This metal ion plays a crucial role in the biological function of numerous enzymes, e.g., carboxypeptidase A, carbonic anhydrase II, and alcohol dehydrogenase. The most commonly encountered structural motif in such enzymes is a tetrahedral zinc coordinated to three histidines and a catalytically important water molecule. As one of the approaches to mimic the cofactor function played by the Zn$^{2+}$ center in biocatalysis, various types of chemical systems with Zn$^{2+}$ complexes based either on the (His)$_3$Zn$^{2+}$($\text{OH}_2$) motif or analogues, e.g., macrocyclic tri- or tetraamines, have been developed. Early approaches to enzyme mimics involved the design of macromolecular receptors such as cyclodextrins bearing appropriately placed functional groups that mimic the amino acid residues known to be involved in catalysis. By using a similar design, calixarene-based biomimetic complexes have been reported.

Cyclodextrins and calixarenes have been attractive as enzyme models because of their enzyme-like properties. Indeed, they possess a hydrophobic pocket capable of binding substrates. In addition, the attachment of an additional binding site for metal ions renders them structurally excellent models for metallo-enzymes, thereby allowing the study of highly specific metal-substrate binding interactions via a cooperative double recognition pattern, jointly connected with metal coordination and hydrophobic substrate binding sites. An essential characteristic of CX4 is that both binding sites are already present (hydrophobic pocket and cation-like receptor), as previously documented for metal ions and noncharged organic molecules. The present work focused on the interaction of the Zn$^{2+}$ ion with geometrically controlled CX4 inclusion complexes of azoalkanes 1, 2 and 3 (Scheme 5-3).

Scheme 5-3. Inclusion geometries of azoalkanes 1 (equatorial), 2 (axial), and 3 (axial).

Interestingly, different effects were observed by the addition of ZnCl$_2$. The large red-shift of the near-UV absorption spectrum of the CX4•1 complex from 367 nm to 353 nm was rather converted to a slight decrease for the CX4•2 complex (Figure 5-2, top), as observed in
neutral condition. The cause of this weak decrease in the UV absorption spectra of 2 in relative release can be explained by a partial immersion of the azo residue in the CX4 cavity, thereby resulting in a weak polarizability. However, the deeper immersion of 3 showed a remarkable decrease which was related to a higher polarizability inside CX4 (Figure 5-2).

**Figure 5-2.** Variation of the near-UV absorption spectra for (a) 2 mM 1, (b) 2 mM 2 in the presence of 4 mM CX4 at pH 7.4 (top) and pH 2.4 (bottom), and 2 mM 3 at pH 7.4 upon addition of increasing amounts of ZnCl$_2$ in D$_2$O.

The control experiment performed with 1 alone in the presence of ZnCl$_2$ suggested the evidence for the formation of a CX4•1•Zn$^{2+}$ ternary complex (Scheme 5-4), in which, as can be seen in the X-ray structure (Figure 5-3), the octahedral Zn$^{2+}$ was coordinated to one of the N atoms of the azo group, which was not the case for substrates 2 and 3. The preferential coordination of the dicationic zinc with atoms N, O or S is well-established in proteins$^{132}$ as well as with synthetic systems.$^{133}$ In addition, the increase of the UV absorbance of chromophores 1 and 2 in acidic media (Figure 5-2, bottom) confirmed the previous findings.
Indeed, the maximum of UV absorption spectrum pointed at 353 nm for 1 provided further evidence for the metal-substrate binding in comparison with 2 and 3, which were rather indicative to a release from the complex upon binding of Zn$^{2+}$ ion to the sulfonato upper rim of CX4 (Scheme 5-4).

**Figure 5-3.** X-ray structure of the CX4•1•Zn$^{2+}$ ternary complex.

![X-ray structure of the CX4•1•Zn$^{2+}$ ternary complex.](image)

**Scheme 5-4.** Supramolecular zinc metallo-enzyme models mimicking structural specificity-dependence of metal-substrate binding interaction.

Based on this observation, a remarkable case of Zn$^{2+}$ coordination upon replacement of the proton of the complexed protonated 1 could be established. The postulated case was strongly supported by the additional Zn$^{2+}$-induced upfield shifted of 0.3 ppm of the H$_{endo}$ proton of 1, while a rather downfield shift in presence the Ca$^{2+}$ was found (Figure 5-4, Table 5-2). Presumably, the guest 1 was deeper immersed in the CX4 hydrophobic cavity, thereby resulting in the formation of a stable complex. Concerning the guest 2, the observed downfield shift of the isopropyl group (Table 5-2) was in good agreement with the expected release monitored spectrophotometrically (Figure 5-2b).
Figure 5-4. $^1$H NMR spectra of CX4•I complexes (2 mM I, 4 mM CX4) in the absence and presence of 50 mM ZnCl$_2$ in D$_2$O.

Table 5-2. Complexation-induced $^1$H-NMR chemical shifts ($\delta$) for the CX4 complexes of I and 2 (2 mM guest, 4 mM CX4) in the absence and presence of 50 mM divalent cations, and binding constants$^a$ (K) of Zn$^{2+}$ in the presence of substrates 1, 2 or 3 in D$_2$O.

<table>
<thead>
<tr>
<th>pD</th>
<th>environment</th>
<th>$\delta$ [ppm]</th>
<th>$K$ [10$^3$ M$^{-1}$]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H$_{endo}$</td>
<td>H$_{exo}$</td>
<td>CH$_3$</td>
</tr>
<tr>
<td>2.4</td>
<td>CX4•I</td>
<td>0.25</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td>+ Zn$^{2+}$</td>
<td>-0.06</td>
<td>-0.03</td>
</tr>
<tr>
<td></td>
<td>+ Ca$^{2+}$</td>
<td>0.56</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>CX4•2</td>
<td>---</td>
<td>0.72</td>
</tr>
<tr>
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<td>+ Zn$^{2+}$</td>
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<td>1.03</td>
</tr>
<tr>
<td></td>
<td>CX4•3</td>
<td>+ Zn$^{2+}$</td>
<td>0.7 ± 0.1$^c$</td>
</tr>
<tr>
<td>7.4</td>
<td>CX4•I</td>
<td>0.15</td>
<td>0.15</td>
</tr>
<tr>
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<td>-0.10</td>
</tr>
<tr>
<td></td>
<td>+ Ca$^{2+}$</td>
<td>0.49</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td>CX4•2</td>
<td>---</td>
<td>1.22</td>
</tr>
<tr>
<td></td>
<td>+ Zn$^{2+}$</td>
<td>---</td>
<td>1.27</td>
</tr>
<tr>
<td></td>
<td>CX4•3</td>
<td>+ Zn$^{2+}$</td>
<td>2.2 ± 0.1$^c$</td>
</tr>
</tbody>
</table>

$^a$ Determined by displacement assay. $^b$ Upon addition of acetylcholine to the CX4•I•Zn$^{2+}$ ternary complex. $^c$ Upon addition of ZnCl$_2$ to the CX4•2 or CX4•3 complex.

The specific I-Zn$^{2+}$ interaction was also evidenced from the steady-state fluorescence quenching behaviour of I in neutral condition (Figure 5-5). Under same conditions, the increase in fluorescence intensity of 2 (Figure 5-6a) and 3 (Figure 5-6b) was related to the quantitative decomposition of the corresponding complex. In line with UV spectral characteristics, the interaction of the divalent cation accompanied by replacement of the proton of the protonated I in acidic solution led to an increase in fluorescence intensity of I, resulting in a weaker quenching when coordinated to Zn$^{2+}$ (inset in Figure 5-5). Note that protonated I is nonfluorescent. In order to assess the stability of the CX4•I•Zn$^{2+}$ ternary
complex, competitive binding experiments with acetylcholine were carried out (Figure 5-6c). The resulting binding constants displayed enhanced binding affinities of Zn\(^{2+}\) ion by a factor of ca. 20 at pD 2.4 and ca. 10 at pD 7.4. The weaker enhancement at pD 7.4 was related to the enhanced cation-π interaction, thereby resulting in a stronger binding of Zn\(^{2+}\) by CX4.

**Figure 5-5.** Fluorescence quenching upon successive addition of ZnCl\(_2\) at pD 7.4. The inset shows the variation in fluorescence intensity of 1 mM 1 in the presence of 1.6 mM CX4 in D\(_2\)O.

**Figure 5-6.** Fluorescence regeneration upon successive addition of (a) ZnCl\(_2\) and (b) acetylcholine at pD 2.4 and pD 7.4. The insets show the increase in relative fluorescence intensity of (a) 1 mM 2 in the presence of 1.6 mM CX4 and (b) 1 mM 1 in the presence of 1.6 mM CX4 and 50 mM ZnCl\(_2\) in D\(_2\)O, fitted according to a competitive binding model.
A related situation as that in the highly specific enzyme-substrate interaction was therefore simplified by our mimetic system (Scheme 5-4). The originality of our concept, which relies on the geometry control of the functional group of bound substrates and the multiple binding ability of artificial receptors like CX4, constitutes an excellent model to obtain insight into the mode of action of metallo-enzymes via the Zn$^{2+}$ ion maintained in their hydrophobic active sites. The inherent effect of the Zn$^{2+}$ bound to DBO substrate in the ternary complex will be further investigated in order to quantify by means of GC-MS technique the photolytic conversion reaction of DBO$^{81,82}$ to bicyclo[2.2.0]hexane and 1,5-hexadiene, as illustrated in Scheme 5-5.

**Scheme 5-5. Photolytic conversion of DBO to bicyclo[2.2.0]hexane and 1,5-hexadiene.**
References


References

(39) Bayer, A. Berlin 1872, 5, 25.
(40) Zinke, A.; Ziegler, E. Berlin 1944, 77, 264.


References


References

References

APPENDICES
Appendix 1

SELECTED PUBLICATIONS
Induced Circular Dichroism and Structural Assignment of the Cyclodextrin Inclusion Complexes of Bicyclic Azoalkanes

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The stoichiometries and binding constants of the host–guest complexes between the bicyclic azoalkanes 1–6 and α-, β-, and γ-cyclodextrins (CDs) and the induced circular dichroism (ICD) of the complexes were analyzed. Assisted by proximity relationships obtained from 2D ROESY NMR spectra, the signs and intensities of the ICD spectra are interpreted in terms of the solution structures (co-conformations) of the CD complexes. The ICD assignments are based on the orientation-intensity ICD rules of Harata and Kodaka, which relate the ICD signs and intensities to the relative orientation of the electric dipole transition moment of the n,π* azo chromophore to the CD axis. The influence of the size of the guest and the host is discussed and the effect of introducing an additional chromophore (either a phenyl or a second azo group) on the ICD spectra is demonstrated.

Introduction

Induced circular dichroism (ICD) is a sensitive spectroscopic tool used to study the solution structures of achiral chromophoric guests with chiral host molecules. Spectral changes caused by the inclusion of guest molecules are frequently more exaggerated in circular dichroism compared to UV–vis spectrophotometry. The method is particularly useful for the structure analysis of natural α-, β-, and γ-cyclodextrins (CDs) as inherently chiral hosts. The structural information obtained from ICD complements the information from X-ray/neutron diffraction and that from NMR techniques, which are restricted in that they refer to the solid state or in that they yield only proximity relationships in solution. Ultimately, a detailed knowledge of the solution structures of host–guest complexes, as can be obtained by ICD, may improve the understanding of molecular recognition phenomena in enzyme–substrate interaction or catalysis, and it may help to advance structure–activity relationships in supramolecular chemistry.

The ICD of bicyclic azo compounds in cyclodextrins has recently been investigated. The fascination for studying such simple cis-azoalkanes arises from the fact that

\[ \text{Equation} \]

the azo group (−N=N−) is one of the smallest and simplest chromophores, but in contrast to the carbonyl group (which could compete in size) it possesses a nonvanishing electric dipole transition moment (“is allowed”), which facilitates interpretations of ICD spectra.

The diazirines7,8,12 and derivatives of 2,3-diazabicyclo[2.2.2]oct-2-ene (1)9–11 which have been examined so far, were sufficiently small to be deeply immersed in their 1:1 host–guest complexes. Moreover, from the direction of the electric dipole transition moment of the azo near UV n,π* transition, which is directed along the azo π orbitals9 (arrows along the y axis in Scheme 1), the solution structures can often be assigned by ICD, 2,3 namely the relative orientation of the guest with respect to the host. The latter has been referred to as the “conformation” of the host–guest complex.13

We have shown9–11 that the rules of Harata1 and Kodaka14 (Scheme 2) can be successfully applied in these cases. This was of considerable interest since previous structural assignments based on ICD have been made for aromatic chromophores with π,π* electronic configuration while that of aliphatic azo compounds is n,π*. In the present work, we selected the bicyclic azoalkanes 1–6 as guest molecules to investigate large structural variations on the complexation by cyclodextrins. In addition, and in contrast to our previous studies, we have also varied the size of the host by using α-, β-, and γ-cyclodextrin, which are composed of 6, 7, and 8 α-d-glucose units.3

### Experimental Section

**Materials.** Compounds 1–5 were synthesized according to literature procedures.15–18 They were purified by sublimation (only 1–4), followed by recrystallization. Compound 6 was a generous gift from Prof. S. F. Nelsen;19 this compound was purified by sublimation before use. α-, β-, and γ-cyclodextrins were purchased from Fluka and used without purification. Deuterium oxide (−99.8%) was purchased from Glaser AG, Basel, Switzerland.

**Spectroscopic Measurements.** All experiments were performed at ambient temperature in D2O. Generally, experiments were performed with 4 mM (ICD) or 2 mM (UV, NMR) solutions of the particular azoalkane. Due to limited solubility, we used only 0.5 mM of 5 and 6 for all measurements. ICD spectra were obtained with a circular dichrograph (0.2-nm resolution, 10 accumulations) by using a blank water–CD solution without guest for background correction. UV spectra were obtained with 0.1-nm resolution.1 H and 2D ROESY NMR spectra were analyzed with the software package MestReC;20 the 2D ROESY spectra were obtained for saturated solutions.

**Computational Studies.** All calculations were carried out with the Hyperchem package.21 The AMBER-S parameterization for saccharides was employed.22 The azoalkanes were kept frozen in the AM1-optimized geometries.

### Results

The complexation behavior of azoalkanes 1–6 by α-, β-, and γ-CD complexes has been analyzed by UV spectrophotometry and by ICD and NMR spectroscopy.11,23 We have consistently employed D2O as solvent, also in the UV and ICD experiments, to be consistent with the NMR studies, and to exclude variances due to solvent isotope effects, which are well-known in the complexation by CDs.24

**Formation of Inclusion Complexes.** The bicyclic azoalkanes show a bathochromic shift in the UV absorption spectra with the enlargement of the cycle,25 e.g., from 340 nm for 2, to 365 nm for 1, to 375 nm for 4 (all in water). Complexation by CD induced also a bathochromic shift, e.g., by ca. 6–8 nm in β-CD-1–4 and by 1.5 nm in β-CD-5, an example is shown in Figure 1. The UV bathochromic spectral shifts of the azo absorption along with the NMR upfield shift of the guest protons, both observed upon addition of CD, signaled the formation of the respective host–guest inclusion complexes. The complexation-induced solvatochromic shift is due to the altered microenvironment, namely the increased polarizability inside the cavity,26 while the NMR shift is characteristic for the shielding by the aliphatic host upon inclusion of the guest.8

2D-NMR ROESY experiments were performed to produce more detailed information on the mode of association.
tion and the inclusion geometry of the complexes with the bichromophoric guests 5 and 6. The 2D ROESY spectra of the β-CD inclusion complex of 6 (mixture of 1:1 and 2:1 complexes) revealed cross-peaks between the H-3 and H-5 protons of CD and all protons of 6, as expected for a deeply immersed symmetrical guest. The 2D-ROESY spectra of the complex 5-β-CD (Figure 2) are in line with a preferential complexation and deep inclusion of the phenyl group; there are strong cross-peaks of the aromatic protons with H-5 and H-6, but the cross-peak with H-3 is much stronger for the ortho than for the meta and para protons, signaling a deep inclusion where the ortho protons reside near H3, cf. Scheme 3. It should be noted that the meta and para proton resonances merge to a multiplet upon complexation, but are separate in the uncomplexed form (500 MHz), thereby allowing unambiguous peak assignments in the aromatic region. The cross-peaks between H-3 and H-5 and the anti and syn protons of the bicyclic substructure of 5 are much weaker, suggesting that the azobicycle is positioned outside or near the upper rim of the CD.

**Stoichiometry of Complexes.** In the majority of the cases, an isosbestic point was observed for the UV titration, e.g., at 372 nm for azoalkane 5 (see inset of Figure 3), which is consistent with a 1:1 complexation mode. Exceptions were the complexation of azoalkane 3 by α-CD and 6 with β-CD, for which the competitive formation of 2:1 complexes (with a single guest and two CDs) could be confirmed through spectroscopic titration plots (see below). The stoichiometry of the complexes was further confirmed through 1H NMR Job plot analysis,27,28 namely for the 5-β-CD and 6-β-CD complexes. For this purpose, the complexation-induced chemical shift of the bridgehead guest protons (ΔδHb) was plotted against the [guest]/([guest] + [host]) concentration ratios. The resulting continuous variation plots (Figure 4) revealed maxima at 0.5 and 0.4, in line with a 1:1 complexation stoichiometry for 5 and a 2:1 complexation pattern for 6. The maximum for 6 falls somewhat above the theoretical value of 0.33,28 which can be rationalized in terms of the small second binding constant compared to the first one (130 versus 17 000 M⁻¹, see Table 1) and the low concentrations employed, which in turn were restricted

**FIGURE 1.** UV titration of azoalkane 3 (2.0 mM) by α-CD. The inset shows the corresponding UV titration plot and fitting at λ = 348 nm according to a 2:1 complexation model (eq 2) for α-CD and according to a 1:1 complexation model (eq 1) for β-CD and γ-CD.

**FIGURE 2.** Diagnostic region of the ROESY NMR spectrum for the β-CD-5 complex.
by the solubility of azoalkane 6 (0.5 mM). Both nonideal conditions can result in a shift of the Job plot maximum away from the theoretical value. Spectroscopic Titrations and Binding Constants.

The complexation-induced UV or NMR shifts were employed to determine the binding constants with $\alpha$, $\beta$, and $\gamma$-CD by spectroscopic titration methods (see inset of Figure 1). In the case of 1:1 complexation, eq 1 was employed in a nonlinear data fitting procedure. In detail, the change in the absorption maximum ($\Delta\varepsilon_{\text{obs}}$, eq 1a, for 1, 2, 3, and 4) or the chemical shift of the particular protons ($\Delta\delta_{\text{obs}}$, eq 1b, for 5) at a constant guest concentra-

<table>
<thead>
<tr>
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<th>$K$ (M$^{-1}$)</th>
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<th>$\beta$-CD</th>
<th>$\gamma$-CD</th>
</tr>
</thead>
<tbody>
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<td>1$^a$</td>
<td>50</td>
<td>1100</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>2$^c$</td>
<td>30</td>
<td>190</td>
<td>$\sim$0</td>
<td></td>
</tr>
<tr>
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<td>70</td>
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<td>4$^c$</td>
<td>10</td>
<td>800</td>
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</tr>
<tr>
<td>5$^f$</td>
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<td>2800</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>6$^c$</td>
<td>$\sim$0$^d$</td>
<td>17000 [130]$^e$</td>
<td>240$^d$</td>
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</tr>
</tbody>
</table>

* The error in the data is 10%. $^a$ From refs 11 and 23. $^b$ UV titration. $^c$ $^1$H NMR titration. $^d$ ICD titration. $^e$ Binding constant for formation of the 2:1 complex given in square brackets.

Complexation of azoalkane 3 by $\alpha$-CD was accompanied by a number of peculiarities, which included the absence of an isosbestic point, the development of vibrational fine structure in the absorption band (Figure 1), and unusually large molar ellipticities (Table 2). The formation of a 2:1 complex needed to be invoked in this case, as well as for complexation of 6 by $\beta$-CD, since the titration plots could not be satisfactorily fitted by eq 1 (Figure 5). A nonlinear fitting procedure according to a 1:1 and 2:1 complexation pattern was used to determine the binding constants $K_1$ and $K_2$ in these cases (Table 1).

Note that the 2:1 fitting process requires the nontrivial numerical solution of the system of coupled equations in eq 2, since an analytical solution is not available. Several programs have been applied for specific examples and a few general programs to solve the problem have also been described. In the present work, we have used both the coupled-equation module implemented in the program ProFit as well as the cubic-equation method to obtain reproducible and self-consistent data. Experimentally, UV-spectrophotometric titration plots (eq 2a) were advantageous for the complexation of azoalkane 3 by $\alpha$-CD due to the large changes in the emerging fine-structured bands (Figure 1), while for the azoalkane 6...

(29) ProFit 5.6.3; QuantumSoft: Zurich, Switzerland.

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β-CD complex ICD titrations produced the best results (eq 2b, Figure 5), because the 1:1 and 2:1 complexes have opposite and therefore very characteristic ICD effects (see below). In both cases, the concentration of guest was kept constant to allow plots of the spectral shifts against the total cyclodextrin concentration, [CD]₀.

\[
\epsilon_{\text{obs}} = \frac{\epsilon_G[G] + \epsilon_{CD,G}[CD\cdot G] + \epsilon_{CD_2,G}[CD_2 \cdot G]}{[G]_0} \quad (2a)
\]

or

\[
\theta_{\text{obs}} = \frac{\theta_{CD,G}[CD\cdot G] + \theta_{CD_2,G}[CD_2 \cdot G]}{[G]_0} \quad (2b)
\]

with

\[
[G] = [G]_0 - [CD\cdot G] - [CD_2 \cdot G]
\]

\[
[CD\cdot G] = \frac{1 + Y + Z - \sqrt{(1 + Y + Z)^2 - 4YZ}}{2K_1}
\]

\[
[CD_2 \cdot G] = \frac{K_2[CD\cdot G][CD]_0 - K_2[CD\cdot G]^2}{1 + K_2[CD\cdot G]}
\]

where

\[
Y = K_1([CD]_0 - [CD_2 \cdot G]) \quad \text{and} \quad Z = K_1([G]_0 - [CD_2 \cdot G])
\]

**Induced Circular Dichroism.** The evolution of the ICD spectra of azoalkanes 1–6 upon addition of α-, β-, or γ-CD was also analyzed to obtain information on the complex geometries. The signs and intensities of the ICD signals as well as molar ellipticities are compiled in Table 2. The absolute intensity of the ICD effects can be directly compared in terms of the molar ellipticity values which extrapolate the observed ICD effects to quantitative complexation. The ICD effects were very different among the various guest molecules (e.g., as shown for β-CD in Figure 6) and the type of CD (e.g., as shown for azoalkane 3 in Figure 7). In the case of azoalkane 3 with α-CD and 6 with β-CD, ICD titrations were performed to obtain independent values for the binding constants and to corroborate the 2:1 stoichiometry and, thus, to complement the results from NMR and UV titrations.

**Force-Field Calculations.** Further insights into the specific modes of inclusion of azoalkanes 1–6 in cyclo-

dextrins were obtained by structure optimizations with the AMBER force field according to our previously described protocol. Force-field calculated structures have been demonstrated, for azoalkane 1 and one bridgehead-alkylated derivative, to be qualitatively consistent with experimental ICD effects, while the quantitative analysis required the consideration of the entire ensemble of energetically accessible conformations. Since we were well aware from the previous studies that the careful evaluation of complex stabilities required more detailed work than the simple energy minimizations available within the scope of the present experimental study, we employed the present calculations merely to

**TABLE 2. ICD Effects and Molar Ellipticities of CD-1–6 Complexes**

<table>
<thead>
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<th>γ-CD</th>
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<td>Δe</td>
</tr>
<tr>
<td></td>
<td>(mdeg)</td>
<td>(mdeg M⁻¹ cm⁻¹)</td>
<td>(mdeg M⁻¹ cm⁻¹)</td>
</tr>
<tr>
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<td>20.0</td>
</tr>
<tr>
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<td>5.0</td>
<td>0.15</td>
<td>16.0</td>
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<tr>
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<td>-11.0</td>
</tr>
<tr>
<td>4</td>
<td>5.0</td>
<td>0.04</td>
<td>3.5</td>
</tr>
<tr>
<td>5a</td>
<td>0.7</td>
<td>0.06</td>
<td>1.7</td>
</tr>
<tr>
<td>6b</td>
<td>3.5 [-0.6]</td>
<td>0.65 [-0.07]</td>
<td>2.5</td>
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</table>

*ICD effect obtained in D₄O with 12 mM host and 4.0 mM guest unless stated differently. * Molar ellipticity calculated as Δe = \(\theta l/c\), cf. ref 37 with \(\theta l/c\). * Independent values for the binding constants and to corroborate the 2:1 stoichiometry and, thus, to complement the results from NMR and UV titrations. * Two signals with opposite signs, cf. Figure 5.

**FIGURE 5.** ICD titration of the bis-azoalkane 6 (0.5 mM) with β-CD. The inset shows the corresponding titration plot and fitting at \(\lambda = 375\) nm according to a 2:1 complexation model (eq 2).

**FIGURE 6.** ICD spectra of azoalkanes 1–4 (4.0 mM) in the presence of β-CD (12 mM).
corroborate experimental findings and interpretations, but refrained from making predictions or assigning co-conformations solely on the basis of force-field calculations.

Some key structures relevant for the present interpretations are shown in Figure 8. Most importantly, the calculations confirmed the high propensity of the gem-dimethyl group in azoalkane 3 to protrude into the α-CD cavity, which is retained in its 2:1 complex (structure a, Figure 8). For azoalkane 6, it is noteworthy that the 1:1 complexation results in a somewhat tilted inclusion geometry, while the formation of the 2:1 complex induces an orientation more parallel to the imaginary axes of the host molecules (structure b, Figure 8). Finally, the phenyl group of azoalkane 5 was shown to have a higher affinity toward complexation than the bicyclic azo group (structure c, Figure 8), and in this case the guest protruded also deepest into the host cavity.

Discussion

We have previously studied the β-CD complexes of azoalkane 1 and its bridgehead-substituted derivatives.9,11 This study has now been extended to the smaller α-CD and the larger γ-CD as host molecules. In addition, we varied the guest by selecting the smaller 2,3-diazabicyclo[2.2.1]hept-2-ene 2 and its 7,7-dimethyl derivative 3, the larger 2,3-diazabicyclo[2.2.3]non-2-ene 4, the 1-phenyl derivative 5, and the bis-azoalkane 6. The azoalkanes 5 and 6 are of special interest since they possess two chromophores, which could respond differently to complexation by the host. Generally speaking, two different modes of complexation appeared viable: a 1:1 complexation with a deep immersion of the entire guest or one chromophoric residue of the guest and a 2:1 complexation in which the guest is "shared" by two CDs.

Monochromophoric Azoalkanes. The monochromophoric derivatives 1–4 and their β-CD complexes will be discussed first. We have previously demonstrated, based on ICD and NMR experiments as well as molecular dynamics calculations, that azoalkane 1 prefers a lateral co-conformation in its β-CD complex (Scheme 4), unless the steric demand of bridgehead substituents induces a frontal co-conformation.9–11 The driving force for formation of the lateral conformation derives from the better hydrophobic interaction for immersing the nonpolar ethano bridge into the cavity as opposed to inclusion of the azo group, which retains interactions with the water molecules near the upper rim region. Note that the electric dipole transition moment in the lateral co-conformation is oriented approximately along the CD axis (Scheme 4). A positive ICD effect is expected in this case (Scheme 2), as experimentally observed (Table 2). The positive ICD effects observed for the β-CD complexes of azoalkanes 2 and 4 (Table 2) can be understood in terms of a lateral co-conformation (Scheme 5) as well, although in these cases it cannot be readily differentiated whether the smaller or larger bridge protrudes into the β-CD cavity. Force-field calculations indicate a higher stability for inclusion of the ethano bridge for both derivatives with β-CD, suggesting that the methano bridge of 2 or the propano bridge of 4 is too small or too large, respectively, to promote a good fit. Note also the
lower binding constants of 2 and 4 relative to 1, which support the improved goodness-of-fit for azoalkane 1.

The negative ICD for the 7,7-dimethyl derivative 3 with \( \beta \)-CD (Table 2) stands out and suggests a significant change of the co-conformation in this complex. The dimethylmethylene group is the most hydrophobic part of the molecule and most likely to protrude into the \( \beta \)-CD cavity, a notion that is confirmed by force-field calculations (Figure 8). There are two alternative interpretations of the observed negative ICD effect of azoalkane 3 with \( \beta \)-CD. The first one would be that a deep immersion may no longer apply for 3 as a consequence of steric repulsion between the dimethylmethylene group and the walls of \( \beta \)-CD, which become narrower when the guest protrudes deeper into the cone-shaped cavity. This could cause a position of the azo group above the upper rim, i.e., outside the inner cavity; Kodaka’s rule (Scheme 2) would then apply, which predicts the opposite ICD sign and could therefore account for the experimental result. This interpretation is disfavored by the fact that the 1:1 inclusion complex of 3 with \( \alpha \)-CD shows a strong positive ICD (see below) even though the \( \alpha \)-CD cavity is smaller and thus should cause the steric effect to become more pronounced, which should force the azo group outside the inner cavity of \( \alpha \)-CD as well. The second interpretation takes into account that the tilt angle of the electric dipole transition moment relative to the CD axis in the lateral co-conformation is small but quite significant (30°, cf. Scheme 4), such that small alterations of the size and substituents on the bridge, which could affect this angle, could well increase this tilt angle and invert the sign of the ICD even for a deep inclusion complex.

The ICD effects for the \( \gamma \)-CD complexes of 1–4 are all weak and positive. Since \( \gamma \)-CD is sufficiently large to form inclusion complexes with all azoalkanes, and since the lateral co-conformation was found to be preferred in \( \beta \)-CD, we propose the same situation for \( \gamma \)-CD as well. Also of interest are the binding constants, which in contrast to the smaller \( \beta \)-CD, follow the order 4 > 1 > 2 as expected from the size and the hydrophobicity of these guests. Note that the smallest and most hydrophilic guest 2 does not show a significant binding with \( \gamma \)-CD at all (Table 1), and therefore produces no ICD signal.

Exclusively positive ICD effects are observed for \( \alpha \)-CD as well, except for azoalkane 6, which is apparently too bulky to bind significantly to this smallest host. The very strong ICD for the 7,7-dimethyl derivative 3 (Figure 7) is particularly noteworthy. In fact, UV and ICD titrations suggest the formation of a 2:1 complex for 3, which did not need to be invoked in any other \( \alpha \)-CD case. For example, an isosbestic point was not observed upon addition of \( \alpha \)-CD to 3 (Figure 1). The complexation of 3 by \( \alpha \)-CD also gave rise to the observation of a fine structure (Figures 1 and 7), which was not observed in the other cases. Hydrogen bonding is known to cause a band broadening of azo absorption bands,\(^{2,6}\) such that the emerging fine structure can be interpreted in terms of an efficient removal of water molecules, e.g., through formation of a tight 2:1 complex.\(^{26}\)

The complexation behavior of azoalkane 3 is reminiscent of the situation for the structurally related molecule camphor,\(^{3,39}\) which forms a 2:1 complex with \( \alpha \)-CD as well. The gem-dimethyl group appears to have a high propensity for complexation with \( \alpha \)-CD on its own, while leaving the remainder of the guest molecule sufficiently exposed to be complexed by a second \( \alpha \)-CD. The prediction of ICD signs for 2:1 complexes cannot be achieved by means of the classical ICD intensity rules for 1:1 complexes (Scheme 2), such that no structural assignments can be reliably based on ICD. Molecular mechanics simulations (Figure 8) do confirm the favorable inclusion of the gem-dimethyl group in both the 1:1 and 2:1 complexes to give rise to the structures depicted in Scheme 6. In the case of camphor, a similar complex structure has been suggested on the basis of NMR longitudinal and transverse relaxation rates for the methyl protons of the corresponding 2:1 complex.\(^{39}\)

**Bichromophoric Azoalkanes.** Azoalkanes 5 and 6 are the largest and most hydrophobic guests, as indicated by their restricted water solubility (ca. 0.5 mM), which accounts for the largest binding constants obtained for the 1:1 complexes of 5 and in particular 6 (Table 1); an exception is \( \alpha \)-CD, which is apparently too small to complex azoalkane 6.

The azo \( n,\pi^* \) absorption band of the phenyl-substituted azoalkane 5 shows a less pronounced bathochromic shift upon \( \beta \)-CD complexation than that of azoalkanes 1–4. In contrast, 5 has a significantly larger binding constant with \( \beta \)-CD (2800 M\(^{-1}\)) than the previously studied derivatives of 1 (ca. 1000 M\(^{-1}\)).\(^{11}\) This enhancement cannot be explained in terms of a change in the co-conformation of the azo chromophore within the cavity since different co-conformers of 1 display similar binding constants.\(^{11}\) Finally, bulky bridgehead substituents are known to promote a frontal alignment and should give rise to a negative ICD effect,\(^{4–11}\) which is not the case for azoalkane 5 (Table 2).

The combined results suggest that the mode of complexation varies for 5 since the more hydrophobic phenyl group preferably protrudes into the cavity. This conclusion is supported by the ICD of the \( \beta \)-CD complex (Figure 3). The ICD spectrum shows a positive signal at 375 nm and below 270 nm, which correspond to the \( n,\pi^* \) band of the azo chromophore and the \( ^1L\alpha \) band of the phenyl group, respectively.\(^{30}\) Preferential binding of the phenyl group results in a complex in which the azo chromophore is located outside the cavity, since the molecular length of 5 (ca. 10 Å) exceeds the depth of CD (8 Å), cf. Scheme 7. This conformational assignment is experimentally confirmed by 2D ROESY NMR experiments, which show


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**SCHEME 6**

![Scheme 6](image)
very strong cross-peaks for the aromatic protons and the inner CD protons. The small bathochromic shift of the azo group in comparison to the other azoalkanes also suggests a position outside the inner cavity. Kodaka’s rule (Scheme 2) therefore applies to the azo chromophore, which predicts a positive ICD effect for the azo group, since the electric dipole transition moment lies outside and is oriented perpendicular to the cavity axis (arrow in Scheme 7). On the other hand, Harata’s rule now holds for the transitions of the deeply immersed aromatic chromophore. The transition moment of the $^1L_e$ transition points along the CD axis, in agreement with the strong positive ICD band in the far-UV region. The azo-group-derived ICD effects for 5 in $\alpha$-CD and $\gamma$-CD are also positive (Table 2) and, therefore, can be explained by assuming similar co-conformations (Scheme 7), but the negative ICD effect of the $\alpha$-CD complex in the far-UV region (ca. 220 nm) remains unaccounted for (Figure 3).

The ICD spectrum of the bis-azoalkane 6 with $\beta$-CD gives rise to a distinct pattern with a very strong positive peak and a weaker negative signal at longer wavelengths (Figure 5). Again, the guest is too large (ca. 10 Å) to be completely immersed such that one or both azo groups may reside partially outside the $\beta$-CD cavity. Motivated by the interpretation of the ICD spectrum of a related bischromophoric azo compound (bis-diazirine), which shows two bands with different signs as well, we considered the possibility that the bands with opposite signs originate from one azo group lying inside the cavity and the other one outside, which could readily explain this pattern due to the expected inversion of ICD signs (Scheme 2); in this case, the bathochromically shifted negative ICD band should correspond to the immersed chromophore due to the characteristic solvatochromic shift of the azo absorption band.

The detailed ICD analysis revealed, however, that this tempting explanation cannot apply for azoalkane 6, because the stronger positive ICD band at 375 nm first increases up to a $\beta$-CD concentration of ca. 1.5 mM, but decreases again at higher concentrations (Figure 5). This decrease of the positive band is accompanied by a bathochromic shift and, more importantly, an emerging weak negative band at longer wavelength. This peculiar titration behavior clearly indicates the presence of a second species, assigned to the 2:1 complex, which emerges at higher $\beta$-CD concentration and gives rise to the negative ICD band. The bathochromic shift of the negative band is in line with this interpretation because the 2:1 complex offers a better protection from the solvent. The strong positive band is consequently assigned to the ICD signal of the 1:1 complex, which decreases with increasing $\beta$-CD concentration. It should be noted that the titration behavior also excludes an interpretation of the observed band splitting in terms of exciton coupling between the two chromophores, which is also not indicated in the UV spectra of the free guest.

The ICD effects for azoalkane 6 cannot be convincingly interpreted in terms of the orientation-intensity rules (Scheme 2), since the complexation geometry of this elongated guest molecule (Scheme 7) places the electric dipole transition moment between a perpendicular and parallel orientation, where definitive assignments cannot be made; these are further complicated by the presence of a second chromophore.

**Conclusions**

In summary, the complexation of bicyclic azoalkanes by cyclodextrins in aqueous solution occurs with medium to weak binding constants, and in two cases the competitive formation of 2:1 complexes could be quantitatively analyzed. The signs and intensities of the ICD effects of the azo $n$,π*$ transition vary strongly with ring size and substitution pattern of the azoalkane as well as with the type of cyclodextrin employed. In limiting cases, the co-conformations of the complexes can be assigned on the basis of Harata’s and Kodaka’s rules, in combination with NMR data. In the majority of cases, the azo compounds appear to adapt a deeply immersed lateral conformation. For bichromophoric systems, competitive complexation of one chromophore and steric effects determine the solution structures of the complexes.

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The complexation of \( p \)-sulfonatocalix[4]arene (CX4) with 2,3-diazabicyclo[2.2.1]hept-2-ene (1), 2,3-diazabicyclo[2.2.2]oct-2-ene (2), 2,3-diazabicyclo[3.2.2]non-2-ene (3), 1-methyl-4-isopropyl-2,3-diazabicyclo[2.2.2]oct-2-ene (4), and 1-phenyl-2,3-diazabicyclo[2.2.2]oct-2-ene (5) was studied in D2O at pH 7.4 by \( ^1 \)H NMR spectroscopy. The formation of deep inclusion complexes was indicated by large upfield \( ^1 \)H NMR shifts of the guest protons (up to 2.6 ppm), which were also used to assign, in combination with 2D ROESY spectra, a preferential inclusion of the isopropyl group of 4 and a dominant inclusion of the azo bicyclic residue for 5. The bicyclic azoalkanes 1–3 showed exceptionally high binding constants on the order of 1000 M\(^{-1}\), 1–2 orders of magnitude larger than for previously investigated noncharged organic guest molecules. The strong binding was attributed to the spherical shape complementarity between the guest and the conical cavity offered by CX4. Interestingly, although the derivatives 4 and 5 are more hydrophobic, they showed a 2–3 times weaker binding, which was again attributed to the deviation from spherical shape in these bridgehead-substituted derivatives. The preferential inclusion of the hydrophilic but spherical bicyclic residue of 5 rather than the hydrophobic aromatic phenyl group provides a unique observation in aqueous host–guest chemistry and corroborates the pronounced spherical shape affinity of CX4.

Introduction

Water-soluble calixarenes of the \( p \)-sulfonato type have been under intense investigation for catalytic, biomimetic, sensor, and separation applications.\(^1\)–\(^{10}\) They are versatile macrocyclic host molecules with metal ion receptor properties, and additionally they provide, depending on their conformation, concave hydrophobic binding sites for organic residues. For \( p \)-sulfonatocalix[4]arene (CX4), a particularly common derivative, electrostatic and cation–π interactions are presumed to be the dominant driving force for complexation; this is reflected in the high binding constants of CX4 with organic (ammonium) cations (10\(^3\)–10\(^5\) M\(^{-1}\)).\(^1\)–\(^{4}\) Binding of noncharged organic guest molecules by CX4 has also been intensively investigated, owing to, among others,

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the potential of water-soluble calixarenes to serve as inverse phase-transfer catalysts. However, the binding constants were consistently found to be undesirably low \((10^{-2} - 10^{-4} \text{ M}^{-1})\), implying a very low hydrophobic driving force for complexation.

The present understanding of the complexation pattern of noncharged organic guests with CX4 in aqueous solution is such that (a) the binding should be weak and (b) the selectivity should be low. We questioned whether the binding constants of noncharged guests could be increased by employing bicyclic molecules, which match the spherical shape of the CX4 cavity in the molecular recognition process. Herein, we selected the azoalkanes, which have recently been established as versatile probe molecules for host–guest complexation phenomena in aqueous solution (e.g., for cyclodextrins and cucurbiturils). They are, in particular, sufficiently water-soluble to obtain direct information on their binding constants and the structures of their complexes with CX4 by \(^1\text{H} \text{NMR spectroscopy.}\)

**Results and Discussion**

**Complexation-Induced Shifts.** Addition of CX4 (0.4–8 mM) to an aqueous solution of 1–5 (0.5–1 mM)


**CHART 1.** Proton Labelling and Calculated Molecular Volumes\(^a\)

<table>
<thead>
<tr>
<th></th>
<th>Hendo/</th>
<th>Hendi/</th>
<th>Hsyn/</th>
<th>Hanti/</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>2</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>3</td>
<td>8 H_C</td>
<td>9 H_C</td>
<td>10 (++)-camphor</td>
<td>10 (-)camphor</td>
</tr>
<tr>
<td>4</td>
<td>8 H_C</td>
<td>9 H_C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8 H_C</td>
<td>9 H_C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Relative magnitudes (uncorrected) of 2D ROESY NMR cross-peaks in the corresponding CX4 complexes are added in parentheses.

at pH 7.4 (adjusted by addition of NaOD) resulted in very large upfield \(^1\text{H} \text{NMR shifts for the azoalkane protons}\) (up to 2.6 ppm, extrapolated to quantitative complexation; Chart 1, Table 1, Figure 1). These are characteristic for the formation of tight and deep host–guest inclusion complexes due to the shielding effect by the aryl groups. \(^4\)–\(^7\),\(^21\),\(^22\) It is well accepted that CX4 adopts a cone conformation in water, the flexibility of which is dependent on pH and the presence of metal ions or organic cations.\(^21\)–\(^23\) The methylene protons of CX4 appear as a broadened singlet at pH 7.4 in the absence of guest, consistent with a flexible cone conformation. The addition of an excess of azoalkane (e.g., 2) resulted in a splitting of this peak (inset of Figure 1), which provides evidence for a rigidification of the cone conformation of the host upon inclusion of the guest (guest template effect).\(^22\) As judged by the averaged complexation-induced shifts, the depth of immersion gradually decreases from 1 (ca. \(-1.9\) ppm) to 2 (ca. \(-1.7\) ppm) to 3 (ca. \(-1.5\) ppm), as expected from the increasing guest size; the calculated van der Waals volumes increased from 95 to 111 to 127 Å\(^3\) for azoalkanes 1–3 (Chart 1).

The complexation-induced shifts for the virtually spherical azoalkanes 1–3 were insufficiently diagnostic to assign the structures of the host–guest complexes in more detail, because the shifts and splitting patterns for the individual bicyclic protons resembled qualitatively those observed upon changing from water to benzene as solvent, except that the shifts in the presence of CX4 were more exaggerated (Table 1). For example, the fact that the exo and anti protons displayed chemically induced shifts 0.3–0.8 ppm larger than those of the endo and syn protons cannot be necessarily interpreted in terms of their deeper immersion into the CX4 cavity, but can be similarly accounted for by the differential response of the chemical shifts toward any aromatic environment (i.e., the same effect is qualitatively observed in benzene as...
TABLE 1. Environment-Induced $^1H$ NMR Shifts Relative to D$_2$O Solution$^a$

<table>
<thead>
<tr>
<th>guest</th>
<th>environment</th>
<th>$H_{endo}$ [ppm]</th>
<th>$H_{exo}$ [ppm]</th>
<th>$H_{syn}$</th>
<th>$H_{anti}$</th>
<th>$H_b$</th>
<th>other H's</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CX4 complex</td>
<td>$-1.44$</td>
<td>$-2.18$</td>
<td>$-1.89$</td>
<td>$-2.61$</td>
<td>$-1.63$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>$-0.23$</td>
<td>$-0.82$</td>
<td>$-0.57$</td>
<td>$-0.96$</td>
<td>$-0.41$</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>CX4 complex</td>
<td>$-1.46$</td>
<td>$-2.10$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-1.25$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>$-0.30$</td>
<td>$-0.75$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-0.30$</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>CX4 complex</td>
<td>$-1.25$</td>
<td>$-1.66$</td>
<td>$-2.04$</td>
<td>$-1.00$</td>
<td>$-$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>CX4 complex</td>
<td>$-0.57$</td>
<td>$-0.54$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-0.17$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>$-0.27$</td>
<td>$-0.65$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>CX4 complex</td>
<td>$-1.49$</td>
<td>$-1.90$</td>
<td>$-$</td>
<td>$-1.96$</td>
<td>$-$</td>
<td></td>
</tr>
<tr>
<td></td>
<td>benzene</td>
<td>$-0.37$</td>
<td>$-0.83$</td>
<td>$-$</td>
<td>$-0.36$</td>
<td>$0.17$</td>
<td></td>
</tr>
<tr>
<td>(+)-camphor</td>
<td>CX4 complex</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Complexation-induced shifts extrapolated to quantitative complexation.

FIGURE 1. $^1H$ NMR shifts of the exo and endo protons of 2 (1 mM) upon addition of 8 mM CX4 at pH 7.4. The inset shows the $^1H$ NMR peak splitting of the methylene protons of CX4 (4 mM) upon addition of 20 mM 2 at pH 7.4.

solvent, where geometrical confinement effects do not apply).

2D ROESY NMR experiments were carried out to obtain complementary information on the inclusion geometries of the CX4 complexes of azoalkanes 1–3 (see relative magnitudes in Chart 1 and Supporting Information). Unlike complexation-induced shifts, ROESY cross-peaks are indicative of specific proximity relationships between host and guest protons (generally 4 Å or less). In agreement with the cone conformation of CX4, the distances of the aromatic host protons to the guest are substantially shorter than those of the methylene protons, such that ROESY cross-peaks with included guest molecules were exclusively observed for the former. For azoalkanes 1–3, NOE enhancements were observed with the bridgehead protons, particularly strong for azoalkane 2, which renders an axial inclusion geometry (with the bridgehead protons pointing toward the open portals) unlikely. Moreover, as observed for azoalkanes 1 and 2 (Chart 1), the cross-peaks for the endo and syn protons were larger than those for the respective exo and anti protons (no cross-peak was detected for the exo proton of 1). These findings suggest that the exo and anti protons and therefore the azo group are located at the upper rim of CX4 near the aromatic host protons, while the exo and anti protons are more deeply immersed, which in turn is entirely consistent with the larger complexation-induced shifts of the latter (Table 1).

The combined NMR evidence for azoalkanes 1–3 suggests an equatorial complex geometry (Chart 2, top), where the polar azo group points toward the aqueous bulk and where the hydrophobic part of the bicycle can efficiently interact with CX4 through CH–π interactions. NMR shift assignments have confirmed similar complexation modes (i.e., with the polar group displaced away from cavity of CX4) for small neutral guests such as ethanol and acetonitrile, as well as substituted benzenes. The equatorial complex structure is also consistent with the relatively moderate polarizability enhancement determined by solvatochromic effects on the absorption spectrum of 2 in its CX4 complex. Due to the location of the azo chromophore, reports more on the polarizability in the region of the hydrated sulfonato groups than near the highly polarizable aryl groups.

For azoalkanes 4 and 5, an axial inclusion mode as that in Chart 2, bottom, is supported by $^1H$ NMR shifts (Table 1). Accordingly, the isopropyl group of 4 protrudes preferentially into the cavity (largest shifts for the isopropyl group), while for 5 the phenyl ring is displaced toward the aqueous bulk (very small shifts for the phenyl protons relative to those of the bicyclic ones). In this

It is noteworthy that the small shifts of the phenyl protons of 5 are not the result of an intrinsically lower response of their chemical shift toward complexation by CX4, since the shifts observed for substituted benzenes upon complexation by CX4 (ca. 1–2 ppm upfield)\(^5,7\) are otherwise known to be as large as those for the bicyclic azoalkanes.

Because the more deeply immersed bridge protons of 4 and 5 are expected to exhibit the larger complexation-induced shifts, the \(^1\)H NMR signals of the exo and exo' (resolved for 4 and 5) as well as the endo and endo' protons (resolved only for 5) were assigned accordingly (Table 1 and Chart 1). The chemical shifts of the bridge protons of azoalkane 4 are also somewhat smaller than those for 2 and 5, which suggests that the immersion of the isopropyl group displaces the bicycle somewhat away from the inner cavity. 2D ROESY NMR experiments further revealed a very strong cross-peak for the exo' proton of azoalkane 4 and a strong one for the isopropyl methyl groups, while for azoalkane 5 a strong cross-peak with the exo proton and a weak one with the endo' proton were observed (Figure 2). All other cross-peaks, in particular those with the bridgehead methyl protons of 4 and with the phenyl protons of 5, were insignificant. The 2D ROESY NMR results are therefore fully in line with the structural assignments based on the complexation-induced shifts (Chart 2).

**Binding Constants.** The host concentration-dependent chemical shifts were employed to determine the binding constants by \(^1\)H NMR titrations. In all cases, the experimental data could be well reproduced by a complexation model based on a 1:1 host–guest stoichiometry (Figure 3), such that higher-order complexes did not need to be postulated. The resulting binding constants for azoalkanes 1–5 were found to be on the order of 1000 M\(^{-1}\) (Table 2).

Bicyclic azoalkanes are very weak bases (\(pK_a \leq 3\))\(^26\) and therefore not protonated in neutral solution.

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**CHART 2. Suggested Structures for the CX4 Inclusion Complexes\(^a\)**

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**FIGURE 2.** Diagnostic regions of the 2D ROESY spectra for the CX4 inclusion complexes of azoalkane 4 (top, 8 mM CX4, 5 mM 4) and azoalkane 5 (bottom, 8 mM CX4, 1 mM 5); asterisk marks an impurity.

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though macrocyclic hosts are known to shift pK\textsubscript{a} values of included guest molecules\textsuperscript{11,27} a protonation of azoalkanes 1–5 in their complexes with CX4 can in fact be experimentally excluded at pH 7.4, since the corresponding UV spectra do not reveal the hypsochromically shifted absorption diagnostic for the protonated azo group, but rather a bathochromic and hyperchromic shift characteristic for the inclusion of the azo group into a more polarizable environment\textsuperscript{25} Electrostatic and cation–π interactions resulting from the formation of protonated guests therefore do not have to be considered in the interpretation of the presently reported binding constants. This renders the binding constants up to 1000 M\textsuperscript{-1} of the bicyclic azoalkanes truly exceptional, since they are 1 order of magnitude larger than those of all but one previously investigated small noncharged organic molecule (Table 2).\textsuperscript{5–8,28}

The first binding constants for “small neutral organic molecules” with CX4 (as opposed to larger polycyclic aromatic hydrocarbons studied by solid–liquid extraction by Gutsche and Alam for a different type of water-soluble calixarenes\textsuperscript{29,30}) were reported by Ungaro and co-workers;\textsuperscript{6} they ranged from 15 to 65 M\textsuperscript{-1} for simple aliphatic ketones, alcohols, and acetonitrile. Schatz and co-workers studied binding of various aromatic compounds with CX4 and observed similarly low binding constants, between 20 and 80 M\textsuperscript{-1}.\textsuperscript{5} Kunsági-Máté et al. have also reached the conclusion, when studying different types of calixarenes, that only very weak complexes with neutral aromatic guests are expected in water.\textsuperscript{9} Kon et al. investigated recently a series of aromatic guests; their observation that electron-deficient aromatic guests display a higher binding (ca. 100 M\textsuperscript{-1}) than their electron-rich counterparts (ca. 10 M\textsuperscript{-1}) provided an indication that π–π electronic interactions, while weak, may be more important in the inclusion of substituted benzenes than unspecific hydrophobic interactions.\textsuperscript{7} Interestingly, a recent investigation of π–π interactions in the complexation of substituted phenols with the larger p-sulfonatocalix[6]-arene (CX6) led to the observation of the opposite electronic substituent effect (i.e., p-nitrophenol formed a weaker complex with CX6 than p-methylphenol).\textsuperscript{31}

In view of the previously reported low binding constants (Table 2), it is appropriate to state that azoalkanes 1–3, in particular, are the first small noncharged organic guests to display a moderately strong binding with CX4; in fact, this substantial binding has provided the basis for a recently introduced fluorescence displacement assay employing azoalkane 2 for monitoring the binding of choline and carnitine derivatives.\textsuperscript{26} The high affinity is the more surprising since azoalkanes 1–3, in particular, are highly water-soluble, much more than the aromatic compounds in Table 2, such that hydrophobic interactions alone are unlikely to be held responsible. Rather, their strong binding reflects an improved goodness-of-fit, unquestionably due to the spherical shape complementarity, which is not fulfilled for the short aliphatic compounds such as ethanol or acetonitrile,\textsuperscript{6} nor for the planar aromatic molecules\textsuperscript{5,7} listed in Table 2. With a little hindsight, one may also note that the equatorial arrangement of the bicycles 1–3 (Chart 2) may optimize CH–π interactions\textsuperscript{24} with all four aryl rings.

To allow further generalization, we have also studied the binding of (+)-camphor as an example of a structurally unrelated yet also bicyclic and spherical guest. The resulting complexation-induced shifts were about as large as those for azoalkanes 1–5 (Chart 1, Table 1), suggesting again the deep immersion of this bicyclic guest. On the basis of the relative magnitudes of the shifts, we tentatively assign a complex geometry, in which the bridgehead proton and the 9-methyl group protrude into the cavity, and where the polar carbonyl group points again to the aqueous bulk (Chart 2). Most importantly, the binding constant was also similarly high (ca. 650 M\textsuperscript{-1}), thereby corroborating the importance of the postulated spherical shape complementarity in the binding of CX4.\textsuperscript{32} For comparison, a similar spherical shape matching, in favor of bicyclic guests over aromatic ones, has been previously inferred for calixarene-based capsules with ammonium ions from mass spectrometry studies.\textsuperscript{33}

However, while the spherical shape of 2, for example, appears to provide a very good match with the CX4 cavity (see above), the binding constants are less critically dependent on the size of the bicyclic system (Chart 1). The choice of the next smaller homologue 1 as guest resulted only in slightly weaker binding, while the next larger one 3 afforded a comparable binding constant, but the binding of (+)-camphor, which exceeds even 3 in size, was slightly lower again (Table 2). This reflects the ability of the conformationally flexible CX4 to adapt the inner conical cavity of CX4 through an induced fit,\textsuperscript{1,2,22} which results in an overall poor to moderate guest size

\textsuperscript{1} Márquez, C.; Nau, W. M. Angew. Chem., Int. Ed. 2001, 40, 3457–3460.
TABLE 2. Binding Constants of Noncharged Organic Guests with CX4 near Neutral pH

<table>
<thead>
<tr>
<th>guest</th>
<th>$K/M^{-1}$</th>
<th>guest</th>
<th>$K/M^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>bicyclic guests</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>aromatic guests</td>
<td>5 (+)-camphor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nitrobenzene</td>
<td>165$^c$</td>
<td>phenylboronic acid</td>
<td>21$^c$</td>
</tr>
<tr>
<td>benzonitrile</td>
<td>91$^d$</td>
<td>iodobenzene</td>
<td>60$^d$</td>
</tr>
<tr>
<td>chlorobenzene</td>
<td>28$^e$</td>
<td>biphenyl</td>
<td>44$^d$</td>
</tr>
<tr>
<td>methylenzalacte</td>
<td>20$^d$</td>
<td>p-chlorobenzonitrile</td>
<td>40$^d$</td>
</tr>
<tr>
<td>benzene</td>
<td>21$^f$</td>
<td>benzyaldehyde</td>
<td>79$^d$</td>
</tr>
<tr>
<td>toluene</td>
<td>20$^f$ [25]$^d$</td>
<td>trans-cinnamaldehyde</td>
<td>78$^d$</td>
</tr>
<tr>
<td>anisole</td>
<td>11$^g$</td>
<td>benzyl tert-butyl nitrooxide</td>
<td>13$^a$</td>
</tr>
<tr>
<td>methanol</td>
<td>no complexation$^h$</td>
<td>1,4-butanol</td>
<td>19$^h$</td>
</tr>
<tr>
<td>ethanol</td>
<td>11$^i$ [32]$^j$</td>
<td>1,5-pentanediol</td>
<td>27$^i$</td>
</tr>
<tr>
<td>2-propanol</td>
<td>22$^k$</td>
<td>acetone</td>
<td>50$^k$ [58]$^k$</td>
</tr>
<tr>
<td>n-propanol</td>
<td>38$^l$</td>
<td>butanone</td>
<td>63$^l$</td>
</tr>
<tr>
<td>n-butanol</td>
<td>43$^m$</td>
<td>acetonitrile</td>
<td>16$^m$</td>
</tr>
<tr>
<td>n-pentanol</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$^a$ This work, determined by $^1H$ NMR titrations at pH 7.4, adjusted with NaOH; average value for different protons; 10% error. $^b$ From ref 25 by UV titration. $^c$ From ref 7 by $^1H$ NMR titrations at pH 7.3 with 0.1 M phosphate buffer. $^d$ From ref 5 by $^1H$ NMR titrations at pH 7.4 with buffer. $^e$ From ref 8 by microcalorimetry at pH 7.5, adjusted with NaOH. $^f$ From ref 6 by $^1H$ NMR titrations at pH 7.3 with 0.1 M phosphate buffer. $^g$ This work, determined by fluorescence regeneration, cf. ref 25, 10% error. $^h$ From ref 28 by EPR spectroscopy, in water.

FIGURE 4. Apparent relationship between the binding constants of bicyclic guests with CX4 and the calculated volume of the guest.

selectivity. For comparison, the binding constants of different tetraalkylammonium ions show also relatively small differences. Apparently, the CX4 cavity can rather ideally provide a spherical space for 8–9 heavy atoms (carbon, nitrogen, or oxygen) or approximately 110–130 Å³ accessible volume (those for 2 and 3), or a cavity diameter of 6.0–6.3 Å (if approximated as a sphere). The “size” of the CX4 “cavity” is accordingly similar to that of β-cyclodextrin (β-CD)$^{18}$ and cucurbit[7]uril,$^{19,34}$ and therefore larger than projected from studies employing aromatic probes.$^7,11$ The bicyclic guest size selectivity is qualitatively reflected in a plot of the binding constants versus the calculated volumes of the guests (Figure 4).

The importance of the spherical shape of 1–3 was corroborated through control experiments with the ellipsoidal derivatives 4 and 5. Although these derivatives are more hydrophobic than the parent 2, they showed a weaker binding (Table 2). It should be noted that the elongated geometry of 4 and 5 requires an axial orientation of the guest inside the complex (Chart 2), which may be less favorable and account for the lower complex stability. For azoalkane 4, the isopropyl group protrudes preferentially into the cavity, as borne out by the relative magnitude of the complexation-induced shifts (Table 1) and 2D ROESY cross-peaks (Chart 2), which this situation is similar to the binding of 4 with β-CD.$^{16,17}$ Note that β-CD shows a comparable binding for 2 and 4 (ca. 1000 M$^{-1}$), suggesting similarly stabilizing intermolecular interactions for these two guests.$^{15–17}$ For azoalkane 5, the complexation-induced $^1H$ NMR shifts as well as the 2D ROESY NMR spectra provide compelling evidence that it is the bicyclic azo residue and not the phenyl group that is included. This inclusion pattern is exactly opposite to that observed for β-CD as host (Chart 3), for which the phenyl group is preferentially included according to 2D ROESY spectral evidence.$^{18}$ In addition, the attachment of the phenyl group (5 versus 2) increases the binding with β-CD by a factor of about 3,$^{18}$ but decreases the binding with CX4 by a factor of 3!

Arimura et al. have previously concluded, on the basis of differential spectral shifts and binding constants reported by a solvatochromic probe, that the cavity of...
CX6, the larger homologue of CX4, is more hydrophobic than β-CD.\textsuperscript{35} Differential hydrophobic interactions were also held responsible for the increased (ca. factor 4) binding of the 1-adamantyltrimethylammonium versus the trimethylanilinium cation with CX4.\textsuperscript{12,21} The present data for binding of azoalkanes 1–5 with CX4, in comparison with β-CD as host, demonstrate that the hydrophobicity of the guest is presumably not the dominant factor for CX4 but rather the spherical shape complementarity.

Particularly instructive is the consideration of the bifunctional guest 5. The phenyl group must unambiguously be considered as the hydrophobic residue, while the bicyclic azo group presents the hydrophilic site. The hydrophilicity of azoalkane 2 is related to its high dipole moment (3.5 D)\textsuperscript{36} and readily demonstrated by simple solubility considerations: The solubility of 2 in water is at least 1% or 100 mM, and its partition coefficient in water/n-pentane and water/diethyl ether is 6.7 and 1.6, respectively, in favor of water. Introduction of the hydrophobic phenyl group dramatically reduces the solubility of 5 to 0.5 mM, yet does not increase, but rather decreases, the binding constant with CX4. In addition, it is not the hydrophobic phenyl group that is preferentially included, as could be projected from the large difference in binding constants between toluene (ca. 20 M\textsuperscript{−1}) and azoalkane 2 (ca. 1000 M\textsuperscript{−1}). The spherical shape complementarity is therefore a sufficiently important criterion to entirely override hydrophobicity considerations in the complexation with CX4. To the best of our knowledge, this is the first example of a complexation process involving a water-soluble macroring host with a hydrophobic cavity in which the inclusion of a hydrophilic spherical guest, in the absence of electrostatic or hydrogen-bonding interactions, is preferred over the inclusion of a similarly sized aromatic hydrophobic residue. A fundamentally different complexation affinity as that well established for cyclodextrins applies. This results in an unconventional inclusion pattern of the amphiphilic azoalkane 5, for which the polar hydrophilic headgroup is buried in the hydrophobic cavity, while the hydrophobic phenyl group remains largely exposed to the aqueous phase.

The question arises why the shape matching is more important for CX4 than for β-CD, although it is well recognized that calixarenes are flexible and should be recognized that calixarenes are flexible and should be better able to adapt to the guest through an induced fit. We presume that the “function” of the guest in the case of CX4 is not only to optimize hydrophobic interactions by minimizing the degree of void space in the host cavity, but also to minimize the repulsive electrostatic interactions between the sulfonato groups. Guests that are too small, such as the aliphatic alcohols and ketones in Table 2 or planar aromatic guests, which may tend to form sandwich-type arrangements between two opposite aryl groups and the guest, may increase hydrophobic or π−π interactions by a contraction of the CX4 cone, but only at the expense of increased electrostatic interactions, thereby resulting in an overall weak stabilization. Bicyclic substrates, on the other hand, appear to be sufficiently large to efficiently fill the inner conical cavity and remove high-energy water but at the same time serve as a spacer to keep all four sulfonato groups well separated. Finally, the “pinched” cone conformation required to optimize π−π interactions with aromatic guests\textsuperscript{37} must be achieved at a substantial entropic cost, the importance of which has recently been emphasized.\textsuperscript{31}

Conclusions

p-Sulfonatocalix[4]arene has been demonstrated to display a relatively moderate guest size selectivity but a strong guest shape selectivity, namely a preference for inclusion of bicyclic (spherical) guests. Although azoalkanes 1–3 are highly water-soluble, they display substantially higher binding constants than previously investigated noncharged organic guest molecules. Most importantly, the spherical shape complementarity over-rides other favorable factors, such as hydrophobic and π−π interactions offered by aromatic guests, which was established through the complexation pattern of the amphiphilic derivative 5. These results question the importance of π−π interactions as a design criterion to optimize the binding of noncharged organic guests with p-sulfonatocalixarenes.

Experimental Section

Commercial p-sulfonatocalix[4]arene (CX4, >97%) and (+)-camphor were used as received. Azoalkanes 1–5 were available from previous studies on complexation with cyclo- dextrins.\textsuperscript{17,18} All experiments were performed at ambient temperature in D\textsubscript{2}O (99.8%). The pD value of the solutions was adjusted by addition of NaOD. pH readings were converted to pD by adding 0.40 units.\textsuperscript{18} 1H and 2D ROESY NMR spectra (400 MHz) were recorded by using the chemical shift of HOD in D\textsubscript{2}O preset at 4.67 ppm as reference. The concentrations for the ROESY spectra varied from 8 to 12 mM CX4 and 1–30 mM azoalkane; they were adjusted to obtain a good peak separation (to avoid overlap for 2 and 3) and sufficient signal strength (lowest for 1 and 3); for 5, the concentration was limited by its low solubility. Molecular volumes were estimated from van der Waals surfaces calculated with the QSAAR module for structures optimized with the MM+ force field,\textsuperscript{39} cf. ref 94.

Acknowledgment. This work was supported by the International University Bremen.

Supporting Information Available: 1H NMR spectral assignments and 2D ROESY NMR spectra for azoalkanes 1 and 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Chiral Resolution through Precipitation of Diastereomeric Capsules in the Form of 2:1 \( \beta \)-Cyclodextrin–Guest Complexes

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Preferential precipitation of one enantiomer from a racemic mixture of a camphane ester of 2,3-diazabicyclo[2.2.2]oct-2-ene was induced by the formation of diastereomeric 2:1 \( \beta \)-cyclodextrin–guest complexes. The precipitate was enriched with the \((-\))-enantiomer and the supernatant solution with the \((+\))-form of a camphane ester, which was quantitatively analyzed in terms of differential binding constants and intrinsic solubilities of the 2:1 complexes. The enantiomeric excess in the precipitate was determined as 30 \pm 3\% by induced circular dichroism.

Cyclodextrins (CDs) are natural macrocyclic oligosaccharide hosts that can enclose small guest molecules in their hydrophobic cavity.\(^1\)\(^\text{2}\) Their inherent chirality, in particular, has led to applications as chiral selectors,\(^3\) as stationary phases in chromatographic separations,\(^2\) or as NMR shift reagents.\(^4\) All of these analytical applications are based on the fact that the inclusion complexes between CDs and enantiomeric guests are intermolecular but diastereomeric in nature, with the associated differences in physical properties.

In contrast to the wealth of applications of CDs to enantioseparation on an analytical scale, mostly chromatography and electrophoresis, there are few reports on the use of CDs for the classical chiral resolution on a preparative scale, i.e., through precipitation, although the diastereomeric complexes are expected to vary in solubility and crystallization behavior. One underlying problem is that CDs have been traditionally used to enhance the solubility of hydrophobic guests, e.g., drugs.\(^5\) The desired host-induced precipitation of the guest is therefore not applicable, but instead a guest-induced precipitation of the host\(^6\) has to be chosen as the route for chiral resolution. This has been documented by Cramer and Dietsche in their pioneering study, which afforded an optical activation of 3–12\% by precipitation from saturated \( \beta \)-CD solutions with a 5–10-fold excess of racemic guest.\(^7\) Methodologically, this less common approach to chiral resolution is not economical, since the (less precious) chiral resolving agent is precipitated with an excess of racemate rather than added to precipitate a dilute racemate. The Cramer method was later employed for partial resolution of chiral phosphinates,\(^8\) sulfanyl compounds,\(^9\) and fenoprofen.\(^10\) The latter X-ray crystallographic study, which revealed enantiomerically enrichment of fenoprofen in 1:1 complexes of \( \beta \)-CD during differential crystal growth on a microscopic scale, has been considered as the only exception of an appreciable partial resolution of a racemate by natural CDs.\(^11\)

Since CD-assisted stereodifferentiation in the solid state from racemate solutions has proven to be difficult for natural CDs, alternative methods have involved synthetic (per)methylated CDs, which are generally considered to be better host molecules for chiral discrimination purposes than natural CDs due to their higher flexibility.\(^11\)–\(^14\) Nevertheless, even with the structurally modified CDs, the achieved ee in the precipitates was found to be less than 30\%, unless repeated precipitation of enantiomerically enriched fractions was pursued,\(^13\)\(^14\) or unless the guest was selected to be in a racemization equilibrium,\(^11\)\(^14\) which presents an impressive yet very special case. In some cases, the enantioseparation relied on differential crystal growth kinetics\(^15\) rather than on intrinsic solubility differences,\(^14\) which further limits routine use.

In short, the use of CDs for chiral resolution through precipitation has been limited to examples employing either an excess of racemate or synthetic CD derivatives. In this Note, we document and quantitatively analyze a differential precipitation of chiral guests that employs unmodified \( \beta \)-CD and exploits the low solubility of higher order (2:1) complexes, in which two host molecules encapsulate a single bifunctional chiral guest.

Results and Discussion

The general concept which we introduce in this Note relies on the formation of precipitating capsules self-assembled from a chiral guest molecule and two chiral host molecules (Scheme 1). The interior of the capsule is decisive for chiral recognition, while the exterior shell imposes a low solubility, which is distinct from previous applications of capsules in chiral recognition. The presently explored method allows for a chiral resolution of racemate solutions solely on account of differential binding constants, while the solubility of the precipitating species may be identical. In other words, the chiral guest serves as a template for the formation of a capsule with low solubility. The precipitating capsules are formed with apparent equilibrium constants \( K_{\text{app}} \), the product of the microscopic binding constants. It is therefore expected, for precipitations on a preparative scale, that the enantiomeric ratio in the precipitate resembles the ratio of the apparent equilibrium constants, which leads to a chiral resolution by preferential encapsulation.

As chiral guest molecules we have selected camphanate esters of the water-soluble azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO), which has recently been established as a versatile nonaromatic probe for CD complexation.20,21 It is accessible to a range of optical spectroscopies, including near-UV absorption and fluorescence, and has been independently established spectrophotometrically by measuring the absorbance of the redissolved precipitate in the near-UV range, where the DBO chromophore has a weak but characteristic absorption band (\( \epsilon \text{ ca. 50 M}^{-1} \text{ cm}^{-1} \)).22 For this purpose, a weighed amount of precipitate was redissolved, and from the UV absorbance (see inset of Figure 1) the host:guest ratio could be determined as 2:1.

The proportion of guest in the precipitate was independently established by NMR spectroscopy. Figure 1 shows the \(^1\)H NMR spectrum of the precipitate obtained by mixing 2 mM (+)-1 and 12 mM \( \beta \)-CD, redissolved in D\(_2\)O with DMF as an internal standard. The integration of the peaks corresponding to \( \beta \)-CD protons between 3.5 and 3.6 ppm (14 H, H-2 and H-3) and the upfield-shifted \( \beta \)-CD gem-dimethyl (1.08 ppm, 6H) suggest a lower solubility of the (+)-form in the presence of \( \beta \)-CD. The precipitates were characterized by NMR spectroscopy. Figure 1 shows the \(^1\)H NMR spectrum of the precipitate obtained by mixing 2 mM (+)-1 and 12 mM \( \beta \)-CD, redissolved in D\(_2\)O containing 2 mM DMF; the inset shows the corresponding UV absorption spectrum of the same solution. Both experiments corroborate that the precipitate contains 1 mM 1 and 2 mM \( \beta \)-CD; see text.

moiety served as a chiral auxiliary, and the esters 1 were prepared from the hydroxymethyl derivative of DBO and camphanic acid chloride in enantiopure as well as racemic form. For reference purposes, we also prepared the individual components, i.e., the enantiopure ethyl camphanates 2 as well as the achiral acetate 3. Precipitation from saturated \( \beta \)-CD solutions (14 mM) was observed when the concentration exceeded 0.38 mM for the (+)-enantiomer and 0.30 mM for the (−)-enantiomer of 1, suggesting a lower solubility of the (−)-form in the presence of \( \beta \)-CD. The precipitates were characterized by NMR spectroscopy. Figure 1 shows the \(^1\)H NMR spectrum of the precipitate obtained by mixing 2 mM (+)-1 and 12 mM \( \beta \)-CD, redissolved in D\(_2\)O with DMF as an internal standard. The integration of the peaks corresponding to \( \beta \)-CD protons between 3.5 and 3.6 ppm (14 H, H-2 and H-3) and the upfield-shifted gem-dimethyl (1.08 ppm, 6H) and bridgehead methyl group (0.96 ppm, 3 H) of the guest revealed the precipitate as a ternary 2:1 \( \beta \)-CD complex.

The proportion of guest in the precipitate was independently established spectrophotometrically by measuring the absorbance of the redissolved precipitate in the near-UV range, where the DBO chromophore has a weak but characteristic absorption band (\( \epsilon \text{ ca. 50 M}^{-1} \text{ cm}^{-1} \)). For this purpose, a weighed amount of precipitate was redissolved, and from the UV absorbance (see inset of Figure 1) the host:guest ratio could be determined as 2:1.

On the basis of the NMR and UV evidence, we concluded that the precipitation was a consequence of the low solubility of the 2:1 inclusion complex. Presumably, the hydroxyl groups of the upper rim, which are important for solubilizing \( \beta \)-CD in water,\(^{23}\) are involved in promoting the binding between the two CD units of the 2:1 complex to form a capsule (Scheme 2), thereby reducing its solubility. Since both moieties of the enantiopure guest (DBO and camphanate) have an intrinsic

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binding affinity with β-CD, quite weak for DBO (K = 320 ± 10 M⁻¹ for 3) and weaker for the (+)-form of 2 (1090 ± 50 M⁻¹) than for the (−)-enantiomer (1320 ± 50 M⁻¹), we can schematically view the complexation process as an initial formation of a 1:1 complex with a binding constant K₁, in which the camphanate moiety preferentially enters the CD cavity (Scheme 2). This is followed by the formation of a 2:1 complex, in which the second moiety (DBO) is complexed by a second CD with a binding constant K₂, eventually followed by precipitation when the solubility limit is exceeded.

To quantify the amount of precipitation, we performed solubility measurements by NMR titrations, i.e., we successively added β-CD to 2 mM guest solutions, and determined the concentrations of guest and host retained in the supernatant. The resulting concentrations are plotted in Figure 2, which reveals the lower solubility of the (−)-enantiomer (Table 1). The double-logarithmic plots are linear (r² > 0.99, Figure 2b), and the slopes are larger than 1 (ca. 1.6), which is in line with the precipitation of a 2:1 complex. In control experiments, no change in the solubility of reference compounds 2 and 3 was observed, suggesting that the individual moieties form soluble 1:1 complexes (complexation-induced shifts) but do not tend to form 2:1 complexes (also confirmed by Job plot analysis) and, therefore, do not precipitate, consistent with Scheme 2. The lower solubility of (−)-1 could be principally due to two reasons: (i) a differential intrinsic solubility of the diastereomeric 2:1 complexes or (ii) different proportions of 2:1 complex in equilibrium resulting from different binding constants K₁ or K₂ or both. To discriminate between these two possibilities, accurate determinations of the microscopic binding constants as well as the intrinsic solubility needed to be performed.

The quantitative analysis of titration plots for sequential 1:1 and 2:1 host–guest binding phenomena is non-trivial even in cases where one concentration (either guest or host) can be kept constant. A closed analytical solution is not available, such that algorithms to solve the related cubic equation are required. In the present case, the analysis became more intricate since both concentrations vary due to the interference of precipitation. We have therefore expanded the algorithm based on the cubic equation by the explicit consideration of two variables, cf. Supporting Information. This allowed the fitting of the complexation-induced shifts from the NMR titrations to afford the equilibrium constants K₁ and K₂. The solubility of 2:1 complex S was obtained in the same fitting procedure from the relationship S = Kᵢ[β-CD-1][1].

All data are listed in Table 1. Also included in Table 1 is the highest concentration of guest, which did not induce precipitation from a saturated β-CD solution, [G]max. The values for K₁ and K₂ have been independently reproduced (within 10% error) by induced circular dichroism and isothermal microcalorimetry titrations in a lower concentration range where no precipitation interfered.

Note that the absolute solubility of the 2:1 complexes S is the same for both enantiomers, within error. The lower solubility of (−)-1 is, however, reflected in the solubility products (Kₛₛₚ), with Kₛₛₚ = [β-CD-1][1] = S/K₂, such that its higher tendency for precipitation can be traced back to differential values of K₂; in addition, the higher K₁ value also favors precipitation of (−)-1, since it increases the 1:1 complex concentration that enters the expression for Kₛₛₚ as well. The product K₁K₂ is therefore decisive for chiral discrimination and reveals a factor of 2 difference (Table 1).

To illustrate the consequences of the differential solubility of the enantiomers on a preparative scale, a precipitation was performed from a 2.0 mM racemic solution of 1 (10 mL) by addition of 5.0 mM β-CD. The circular dichroism spectra of the redissolved precipitate and the supernatant solution (Figure 3) immediately indicated that the solution had been enriched with the (+)-enantiomer and the precipitate with the (−)-form, in line with the projected solubility data (note that the circular dichroism spectra are not symmetric due to the presence of β-CD, which contributes an induced circular dichroism).

TABLE 1. Binding Constants and Solubility Parameters for Enantiomers 1 in the Presence of β-CD in D₂O

<table>
<thead>
<tr>
<th>guest</th>
<th>K₁ (M⁻¹)</th>
<th>K₂ (M⁻²)</th>
<th>Kₛₛₚ (10⁻⁶ M⁻²)</th>
<th>S (mM)</th>
<th>[G]max (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-</td>
<td>1200 ± 180</td>
<td>130 ± 10</td>
<td>156 000</td>
<td>1.7 ± 0.3</td>
<td>0.22 ± 0.03</td>
</tr>
<tr>
<td>(−)-</td>
<td>1590 ± 190</td>
<td>210 ± 20</td>
<td>333 900</td>
<td>1.0 ± 0.2</td>
<td>0.21 ± 0.04</td>
</tr>
</tbody>
</table>

Solubility of 2:1 complex. Maximum (experimental) concentration [G]max. The values for K₁ and K₂ have been independently reproduced (within 10% error) by induced circular dichroism and isothermal microcalorimetry titrations in a lower concentration range where no precipitation interfered.

CDs are employed, (ii) the guest to be enantiomERICALLY enriched can be used as a limiting reagent, and (iii) the enantiomeric enrichment is predictable on the basis of the binding constants \((K_1K_2)\) product. In terms of the general concept described in the outset, we have formed capsules of limited solubility, namely, 2:1 CD complexes. We attribute the lower solubility of the capsules to the formation of intermolecular hydrogen bonds between the two hosts, which are thus no longer available to promote solubilization through hydrogen bonding with water, as is possible for the free CD and 1:1 CD complexes. A similar approach may therefore be applicable to other hydrogen-bonded capsules.\(^{19}\)

In summary, we have analyzed in detail an intricate equilibrium involving the formation of 1:1 and 2:1 CD complexes with an enantiomERIC guest displaying a concomitant precipitation of the 2:1 complex. The differential binding constants for 1:1 as well as 2:1 complex formation result in a lower solubility of one enantiomer in the presence of \(\beta\)-cyclodextrin. This can be exploited to achieve a chiral resolution by precipitation of the diastereomeric 2:1 complexes, as evidenced by circular dichroism spectroscopy.

### Experimental Section

**Materials.** The commercial materials 1R(+)- and 1S(−)- camphamic acid chloride (> 98%), \(\alpha\)-cyclodextrin (> 98%), acetyl chloride (> 99%), and silica gel 60 were used as received. 1-Hydroxyethyl-2,3-diazabicyclo[2.2.2]oct-2-ene was synthesized according to a literature procedure.\(^{17,18}\) D2O (> 99%) was used as solvent for all spectroscopic measurements.

**General Procedure for Esterification of Alcohols.** The respective acid chloride (2 mmol) in 3 mL of anhydrous pyridine was added dropwise to a stirred solution of the corresponding alcohol in 3 mL of anhydrous pyridine (1.5 mmol) containing a catalytic amount of 4-(dimethylamino)pyridine at 0 °C under a dry nitrogen atmosphere. The mixture was stirred for 2 h and then extracted and washed three times, first with 10 mL of a 10% sodium hydrogen carbonate solution and then twice with 10 mL saturated aqueous NaCl. The organic phase was dried and concentrated under reduced pressure. Subsequent purification by silica gel chromatography (98:2 CH3Cl/MeOH) followed by 3-fold recrystallization from n-hexane provided the purified ester. The compound characterization data for (+)-I are given below; those for (−)-I, 2, and 3 are given in Supporting Information.

\((+)-\text{I}.\) Yield 68%, mp 86–87 °C; UV (H2O) \(\lambda_{\text{max}}=366\text{ nm}, \epsilon = 57 \text{ M}^{-1}\text{ cm}^{-1}\); circular dichroism (D2O) \(\lambda_{\text{max}}=370\text{ nm}, \Delta \epsilon = 0.16 \text{ M}^{-1}\text{ cm}^{-1}\); \(\lambda_{\text{max}}=226\text{ nm}, \Delta \epsilon = 0.30 \text{ M}^{-1}\text{ cm}^{-1}\); \(^1\text{H} \text{ NMR (300 MHz, CDCl}_3\)} @ 0.99 (s, 3H), 1.07 (s, 3H), 1.12 (s, 3H), 1.15–1.42 (m, 4H), 1.56–1.74 (m, 5H), 1.88–1.98 (m, 1H), 2.02–2.11 (m, 1H), 2.42–2.52 (m, 1H), 4.78 (dd, 2H, J 6.36, 11.43), 5.18 (bs, 1H); \(^{13}\text{C} \text{ NMR (75 MHz, CDCl}_3\)} @ 9.9, 17.0, 21.5, 23.6, 29.1, 30.9, 31.0, 54.5, 55.0, 61.8, 65.6, 69.8, 91.4, 167.6, 178.4; FAB-MS m/z 321 (\(M + H^+\)). Anal. Caled for C17H24N2O4: C, 63.73; H, 7.55; N, 8.74; O, 19.97. Found: C, 63.82; H, 7.63; N, 8.70; O, 19.95.

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**Supporting Information Available:** Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Chiral discrimination in the complexation of heptakis-(2,6-di-O-methyl)-β-cyclodextrin with 2,3-diazabicyclo[2.2.2]oct-2-ene derivatives

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Abstract

The chiral discrimination of enantiomeric camphanate esters of 2,3-diazabicyclo[2.2.2]oct-2-ene by heptakis-(2,6-di-O-methyl)-β-cyclodextrin was studied by means of induced circular dichroism, UV spectrophotometry, and fluorescence spectroscopy. The first two spectroscopic techniques were employed to study the thermodynamics, while the kinetics of complexation was determined by using steady-state fluorescence quenching experiments. The formation of 1:1 and 2:1 inclusion complexes was monitored through opposite induced circular dichroism effects and an increase of the near-UV extinction coefficient of the azo chromophore, from which the binding constants \( K \) were determined by means of titrations. The binding constants for 1:1 complexation (ca. 1500 M\(^{-1}\)) were more than 1 order of magnitude larger than those for 2:1 complexation (ca. 40 M\(^{-1}\)). An insignificant chiral discrimination was found for the thermodynamics of 1:1 complexation, but a significant effect on the association kinetics, which was ca. 20% faster for the \((-)\)-enantiomer. The association rate constants for the formation of the 2:1 complex were found to be too small (<1 × 10\(^7\) s\(^{-1}\)) to allow determination by the fluorescence quenching method.

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Keywords: Chiral discrimination; Cyclodextrins; Fluorescence; Circular dichroism; Host–guest complexes

1. Introduction

Cyclodextrins (CDs) are water-soluble, naturally occurring container-type host molecules, which are able to include a variety of guest molecules [1]. Their host–guest complexation behavior has been studied in great detail [2,3], which has assisted the development of applications in biomimetics [4], photochemistry [5], drug discovery [6], catalysis [7], and analytical techniques [8]. Owing to their natural occurrence as a single enantiomeric form, their complexation with racemic guests leads to the formation of diastereomeric complexes displaying different physical properties. For example, the complexes display different chemical shifts, which has enabled the use of CDs as chiral shift reagents in NMR spectroscopy [9,10].

Structural differences between diastereomeric CD complexes have been analyzed in detail from crystallographic data in the solid state [11], while induced circular dichroism, which has been frequently employed for structural assignments of CD complexes in solution [12-14], has been less frequently employed to study structural differences in diastereomeric CD complexes. Thermodynamic data are more readily accessible, including binding constants for diastereomeric complexes [3]. Temperature-dependent measurements or, preferably, isothermal titration calorimetry, have afforded insights into enthalpic versus entropic factors, which has been used to track the origin of the enantioselectivity for the complexation of chiral guests with CDs [15]. Note that different binding constants of enantiomers enable the use of CDs as chiral selectors in chromatography-based separation techniques [8]. In contrast to thermodynamic differences of diastereomeric host–guest complexes, differences in the complexation kinetics (rate constants for association and dissociation) have been rarely considered, although they could be equally important in applications relying on reversible, dynamic processes like those found in chromatography for the exchange between stationary phase and analyte, for catal-

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In this work, we choose the (+)/(−)-camphanate esters of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) as a new versatile probe for assessing supramolecular complexation phenomena [17].

We have recently introduced the azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) as a new versatile probe for assessing supramolecular complexation phenomena [17]. In addition to its high water solubility and non-aromaticity, which differentiates it from traditional photophysical probes [21,36], DBO is accessible by near-UV absorption and induced circular dichroism as well as fluorescence spectroscopy [13,17,18,37,38]. While the first two techniques, along with 1H NMR, allow reliable assessments of the thermodynamics of complexation, its exceedingly long-lived fluorescence (505 ns in aerated D2O) [18] has been used to probe the kinetics of complexation with CDs by time-resolved as well as steady-state fluorescence quenching [17,18], and indirect external quencher experiments [16,19,27]. Alternatively, indirect techniques are based on NMR coalescence or spin exchange spectroscopy (slow kinetics) [28,29], or on EPR coalescence (faster kinetics) [30,31]. Stopped-flow methods have also been applied in some cases [32–34].

Nowadays, there is an agreement that the association rate constants of uncharged polar as well as nonpolar guest molecules are on the order of 10^5–10^6 M^-1 s^-1, typically about a factor of 10 below the diffusion-controlled rate, if the guest molecules are sufficiently small to form tight inclusion complexes with the selected CD [16–19,23–27,31]. Slower rates may apply if the selected CD is small (mostly for α-CD) and the selected guest is too large [22,32,33], or if higher order (2:1 and 2:2) complexes are formed [34]. Substantially slower rate constants have been reported in the literature, and in some cases it is not clear whether the slow rate constants are real, e.g., due to the presence of charges [28,29,32], whether they are related to the model-dependent indirect data analysis [30], or whether they report on a secondary relocation reaction involving pre-equilibria rather than on the elementary process of primary association [32,33]. The dissociation rate constants show naturally a larger variation [18,19], since they depend inversely on the binding affinity of the guests, which shows five orders of magnitude variation among different guests (K = 10^6 M^-1 to K = 10^4 M^-1) [2,35].

In addition to its high water solubility and non-aromaticity, which differentiates it from traditional photophysical probes [21,36], DBO is accessible by near-UV absorption and induced circular dichroism as well as fluorescence spectroscopy [13,17,18,37,38]. While the first two techniques, along with 1H NMR, allow reliable assessments of the thermodynamics of complexation, its exceedingly long-lived fluorescence (505 ns in aerated D2O) [18] has been used to probe the kinetics of complexation with CDs by time-resolved as well as steady-state fluorescence quenching [17,18]. In addition, the induced circular dichroism effects have been employed to determine the co-conformations of the host–guest complexes in solution [13,37,39], i.e., the relative coconformation of the host to the guest inside the CD cavity [40]. In this work, we choose the (+)/(−)-camphanate esters of 2,3-diazabicyclo[2.2.2]oct-2-ene (1) as enantiomeric guest molecules to investigate chiral discrimination by UV absorption, induced circular dichroism, and fluorescence spectroscopy in the search for overarching relationships between structural, thermodynamic, and kinetic aspects of the molecular recognition process. While the DBO chromophore served mainly as photophysical probe, the camphanate moiety was selected as chiral auxiliary, since camphor and its derivatives have been shown to be responsive to chiral discrimination by CDs [15,41]. In the present study, chiral discrimination of the kinetics of CD complex formation was of particular interest, since a previous approach toward this goal was not successful [16]. Amongst the various CDs with different sizes, β-CD was selected, which shows the highest binding constant with the DBO probe [17]. Preliminary experiments were performed with natural β-CD as host, but this complexation was complicated by precipitation of the 2:1 complex, which is described separately [38]; the synthesis of the chiral esters 1 has also been documented in this context. Herein, instead of the natural β-CD, the derivative heptakis(2,6-di-O-methyl)-β-cyclodextrin (DMe-β-CD) was employed, in which the 2-OH and 6-OH groups are methylated, see structure below. DMe-β-CD is also better water-soluble than β-CD, which suppressed precipitation of its complexes with 1 and allowed additionally the use of higher host concentrations, e.g., to more reliably determine higher-order equilibria.

2. Experimental details

2.1. Materials

DMe-β-CD (> 98%) was purchased from Fluka and used without further purification. The esters 1 were prepared from the hydroxymethyl derivative of DBO and the camphanic acid chlorides in enantiopure form as previously described [38]. All measurements were performed in aerated D2O (Glaser AG, Basel, Switzerland, >99%).
2.2. Spectroscopic measurements

Circular dichroism spectra were recorded with a Jasco J-810 circular dichrograph (0.1 nm resolution, 5 accumulations) with reference to a D₂O solution containing the same host concentration, but without added guest. UV spectra were obtained with a Varian Cary 4000 spectrophotometer (0.1 nm resolution). Corrected steady-state fluorescence spectra were measured with a Varian Cary Eclipse fluorometer. Steady-state fluorescence quenching experiments with DMe-β-CD were performed with excitation at 367 nm, near the isosbestic point between free DBO and its 1:1 complexes. All titration experiments were performed in conventional 4-mL Hellma fluorescence quartz cells (light path 10 mm) by using 2.5 mL of a solution containing 0.5–2 mM guest and successively adding solid host (0.1–50 mM).

3. Results and discussion

3.1. UV absorption measurements

The effect of the addition of DMe-β-CD on the UV absorption spectra of I was studied in the wavelength range between 300 and 400 nm by keeping the concentration of I constant at 2 mM and varying the concentration of DMe-β-CD from 0.2 to 50 mM. As a typical example of the observed spectral change with the addition of host, the UV spectra of the (−)-enantiomer at various DMe-β-CD concentrations are shown in Fig. 1. In the presence of DMe-β-CD, the absorption maximum of the azo chromophore (λ_max = 366 nm, ε = 57 M⁻¹ cm⁻¹) undergoes a batho- and hyperchromic shift (λ_max = 377 nm, ε = 77 M⁻¹ cm⁻¹ at 50 mM DMe-β-CD). The well-defined isosbestic points at about 367 nm (at low host concentration) and 373 nm (at high host concentration) are indicative of a three-state equilibrium with sequential complexation and significant differences in the binding constants. This can be assigned to the initial formation of a 1:1 complex at low concentration followed by the formation of a 1:2 complex at higher concentration (Scheme 1, lower reaction sequence). The binding constant for 2:1 complexa-
tion \((K_2)\) must therefore be substantially smaller than for 1:1 complexation \((K_1)\); otherwise, the isosbestic points would be less well defined or absent. Very similar behavior with minor spectral differences was found for the \((+)-\)enantiomer.

In Scheme 1, we assume that the camphanate moiety of 1 is preferentially included in the 1:1 complex, and that the second host molecule subsequently caps the DBO part. This sequence appears reasonable on the basis of the binding constants, which were found to be larger for the respective model compounds \((\approx 1200 \text{ M}^{-1} \text{ for } (+)/(-)-\text{ethyl camphanate and } 320 \text{ M}^{-1} \text{ for acetylated 1-hydroxymethyl–DBO})\) [38], and is also supported by the spectroscopic data, which suggest a greatly altered microenvironment for the DBO chromophore in the 1:2 versus the 1:1 complex, e.g., a larger bathochromic shift.

The absorption-spectral response of the DBO chromophore to the environment is well understood [42,43]. Accordingly, the bathochromic shift upon addition of DMe-\(\beta\)-CD, which is accompanied by an enhanced oscillator strength \((P)\), which the DBO chromophore experiences inside the 2:1 inclusion complex [42]. A value of \(P=0.235 \pm 0.005\) can be extrapolated to conditions of quantitative complexation, which is somewhat larger than the polarizability found inside 1:1 complexes \(P=0.204\) [43], in line with the better protection from the aqueous bulk.

3.2. Circular dichroism and induced circular dichroism measurements

The circular dichroism spectra of both enantiomers in the absence of DMe-\(\beta\)-CD (Fig. 2) display a virtually perfect mirror–image relationship, which confirms the purity of the newly synthesized samples in general, and their enantiomeric purity in particular. There are two circular dichroism bands centered at 226 nm and a weaker one at 370 nm. The former is related to the \(n, \pi^*\) Cotton effect of the carboxyl group, while the latter is characteristic for the \(n, \pi^*\) transition of the azo chromophore [13,39]. Note that the azo transition responds quite strongly with respect to its chiro-optic properties to the remotely tethered chiral auxiliary.

Fig. 3 shows the circular dichroism spectra of 1 at varying DMe-\(\beta\)-CD concentrations. The concentration of 1 was kept constant at 2 mM, and the circular dichroism spectra were measured at increasing DMe-\(\beta\)-CD concentrations from 0.2 to 50 mM. The addition of host causes highly significant and impressive effects on the circular dichroism spectra, such that we rate the information afforded by these titrations as particularly reliable. The most remarkable change in the presence of DMe-\(\beta\)-CD is the intensity and sign of the band peaked out at 370 nm. In the case of \((+)-1\), the addition of increasing amounts of DMe-\(\beta\)-CD causes first a decrease in intensity of this band and then a more pronounced increase with a red shift resulting in a maximum around 374 nm.

The effect can be corrected by subtracting the intrinsic circular dichroism effect of uncomplexed \((+)-1\). This affords the induced circular dichroism caused by complexation (inset in Fig. 3a), which reveals a down-and-up spectral feature, where the negative effect at low concentrations...
can again be assigned to the circular dichroism induced in the 1:1 complex, and the positive effect to that induced in the 2:1 complex (Scheme 1, lower reaction sequence). The bathochromic shift of the band in the 2:1 complex is independently supported by the UV-spectral behavior (see above). The induced circular dichroism behavior of the (−)-enantiomer is qualitatively opposite (Fig. 3b), i.e., a positive effect is observed at low concentrations, followed by a negative effect. The spectral features in the presence of host are, however, not perfectly symmetrical to each other, since the resulting host-guest complexes are diastereomeric in nature and have slightly different structures and chiro-optic properties (in addition to opposite signs).

As predicted by the rules of Harata and Kodaka [12,44], the signs of the induced circular dichroism effects are principally suitable to provide information on the co-conformation of CD complexes with chromophoric guest, which has recently been confirmed for azanaphthosilane guests [13,18,37,39]. Unfortunately, these rules have been formulated for achiral guest molecules, which lack an intrinsic circular dichroism, and they cannot be transferred in a straightforward manner to the inclusion complexes with chiral guests. The opposite signs when the azo chromophore is positioned outside the CD cavity. It is therefore reasonable to expect different signs when the azo chromophore is positioned outside the CD cavity (1:1 complex) or inside the CD (2:1 complex), and they cannot be transferred in a straightforward manner to the inclusion complexes with chiral guests. The opposite signs have also been described [47,48]. In the present work, we have used the cubic-equation module implemented in the program ProFit [49] to obtain reproducible and self-consistent results. In detail, the spectral changes in the UV absorbance (Eq. (1a); inset of Fig. 1) or the ellipticity (Eq. (1b); Fig. 4) were plotted against the DMMe-β-CD concentration, [CD] 0 , to afford the binding constants in Table 1.

In both cases, the total concentration of the guest, [I] 0 , was held constant at 2 mM, and the titrations were performed at the wavelengths of maximum change, i.e., 370 nm for circular dichroism, and 377 nm for UV absorption; the path length (l) was 1 cm in all experiments.

For the cubic equation of a DMMe-β-CD concen-

\[
\begin{align*}
\Delta \varepsilon & = \epsilon_1 (1) + \epsilon_{CD} (CD \cdot I) + \epsilon_{CD,2} (CD_2 \cdot I), \\
\phi & = \frac{32982}{337} \Delta \varepsilon_1 (1) + \Delta \varepsilon_{CD} (CD \cdot I) + \Delta \varepsilon_{CD,2} (CD_2 \cdot I),
\end{align*}
\]

with

\[
\begin{align*}
[I] &= \frac{[I]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD] &= \frac{[CD]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD_2] &= \frac{[CD_2]_0}{K_1 ([CD] + K_2 [CD]^2)}
\end{align*}
\]

The complexation-induced circular dichroism and UV-spectral changes can be used to determine the binding constants K1 and K2 for 1:1 and 2:1 complexation, respectively. Fitting of the titration data to the sequential complexation model requires a numerical solution (an analytical solution is not available). Several programs have been applied for specific examples [45,46], and a few general programs to solve the problem have also been described [47,48]. In the present work, we have used the cubic-equation module implemented in the program ProFit [49] to obtain reproducible and self-consistent results. In detail, the spectral changes in the UV absorbance (Eq. (1a); inset of Fig. 1) or the ellipticity (Eq. (1b); Fig. 4) were plotted against the DMMe-β-CD concentration, [CD] 0 , to afford the binding constants in Table 1.

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\phi & = \frac{32982}{337} \Delta \varepsilon_1 (1) + \Delta \varepsilon_{CD} (CD \cdot I) + \Delta \varepsilon_{CD,2} (CD_2 \cdot I),
\end{align*}
\]

with

\[
\begin{align*}
[I] &= \frac{[I]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD] &= \frac{[CD]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD_2] &= \frac{[CD_2]_0}{K_1 ([CD] + K_2 [CD]^2)}
\end{align*}
\]

The complexation-induced circular dichroism and UV-spectral changes can be used to determine the binding constants K1 and K2 for 1:1 and 2:1 complexation, respectively. Fitting of the titration data to the sequential complexation model requires a numerical solution (an analytical solution is not available). Several programs have been applied for specific examples [45,46], and a few general programs to solve the problem have also been described [47,48]. In the present work, we have used the cubic-equation module implemented in the program ProFit [49] to obtain reproducible and self-consistent results. In detail, the spectral changes in the UV absorbance (Eq. (1a); inset of Fig. 1) or the ellipticity (Eq. (1b); Fig. 4) were plotted against the DMMe-β-CD concentration, [CD] 0 , to afford the binding constants in Table 1.

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\phi & = \frac{32982}{337} \Delta \varepsilon_1 (1) + \Delta \varepsilon_{CD} (CD \cdot I) + \Delta \varepsilon_{CD,2} (CD_2 \cdot I),
\end{align*}
\]

with

\[
\begin{align*}
[I] &= \frac{[I]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD] &= \frac{[CD]_0}{K_1 ([CD] + K_2 [CD]^2)}, \\
[CD_2] &= \frac{[CD_2]_0}{K_1 ([CD] + K_2 [CD]^2)}
\end{align*}
\]
2:1 complex ($K_2$). Although the absolute values of the two independent titration methods vary slightly, both suggest a 20% higher $K_1$ value for complexation of the (+)-enantiomer. The binding constants for the $K_2$ value show the opposite trend (larger value for (−)-enantiomer). Unfortunately, the error limits of the fitted binding constants prevent affirmative conclusions. The errors in $K_1$ and $K_2$ are also correlated, i.e., the choice of a larger value of $K_1$ can be compensated by a smaller value of $K_2$ and vice versa, which somewhat limits intuitive interpretations based on these error limits. An alternative parameter, which better reflects error-correlation, is the product of both binding constants $K_1 K_2$, which is also listed in Table 1. We consider the binding constants obtained from circular dichroism as being more reliable than the UV data for two reasons. First, the circular dichroism titrations show a characteristic inflection point with large absolute variation (Fig. 4), which reduces the error in the fitting (in particular for the $K_1 K_2$ product), and second, the method is insensitive to background absorption due to achiral absorbing impurities.

The binding constants $K_1$ and $K_2$ afforded by the two techniques suggest an insignificant chiral discrimination for the thermodynamics of guest binding. The diastereomeric complexes show, however, distinct spectroscopic properties, e.g., a 5% variance in the extrapolated extinction coefficients (from Eq. (1a)) and different fluorescence lifetimes (see below) of the 2:1 complexes (Table 1), which suggest a slight change of the inclusion co-conformations.

### 3.4. Steady-state fluorescence quenching

The DBO chromophore is quenched inside CD cavities, presumably by hydrogen atom abstraction [17,18,24], which was herein exploited to probe the kinetics of complexation of I with DMe-C-CD. The conceptual basis for the measurement of association rate constants by fluorescence quenching is therefore more efficient than expected from the ground-state equilibrium distribution of the complexed and uncomplexed forms alone. In other words, the fluorescence quenching is partially dynamic, and not only static, in nature, which provides a spectroscopic handle on the kinetics. Surprisingly, and in contrast to aromatic fluorescent probes, substitution of DBO shows a tendency to prolong the fluorescence lifetimes rather than to shorten them [18], and this was also the case for the camphanate derivatives I, which show a longer lifetime in D$_2$O (620 ns aerated, 840 ns deaerated, this work) than the parent DBO (505 ns aerated, 730 ns deaerated) [17,18]. The conceptual requirement of a long fluorescence lifetime is therefore fulfilled, and in fact improved.

The fluorescence quenching of DBO-type azoalkanes can be followed by time-resolved as well as steady-state fluorescence decays (which become more complex for a 2:1 than for the previously investigated 1:1 complexation scheme) and focused on an analysis of the steady-state fluorescence behavior. The latter can also

### Table 1

Thermodynamic, kinetic, and spectroscopic parameters for the complexation of the enantiomers I with DMe-β-CD in D$_2$O under air

<table>
<thead>
<tr>
<th>Parameter</th>
<th>From circular dichroism</th>
<th>From UV absorption</th>
<th>From circular dichroism</th>
<th>From UV absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_1$ (M$^{-1}$)</td>
<td>1440 ± 180</td>
<td>1790 ± 200</td>
<td>1190 ± 140</td>
<td>1390 ± 260</td>
</tr>
<tr>
<td>$K_2$ (M$^{-1}$)</td>
<td>36 ± 2</td>
<td>34 ± 4</td>
<td>34 ± 4</td>
<td>34 ± 4</td>
</tr>
<tr>
<td>$K_1 K_2$ (M$^{-2}$)</td>
<td>52000 ± 4000</td>
<td>35000 ± 8000</td>
<td>52000 ± 3000</td>
<td>37000 ± 15000</td>
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</table>

Kinetic parameters:

<table>
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<th>Value</th>
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<tr>
<td>$k_{obs}$ (10$^5$ M$^{-1}$ s$^{-1}$)</td>
<td>1.12 ± 0.03</td>
</tr>
<tr>
<td>$k_{obs}$ (10$^5$ M$^{-1}$ s$^{-1}$)</td>
<td>0.87 ± 0.02</td>
</tr>
<tr>
<td>$k_{obs}$ (10$^5$ M$^{-1}$ s$^{-1}$)</td>
<td>1.28 ± 0.06</td>
</tr>
<tr>
<td>$k_{obs}$ (10$^5$ M$^{-1}$ s$^{-1}$)</td>
<td>1.12 ± 0.05</td>
</tr>
</tbody>
</table>

### Spectroscopic parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta M$ (nm$^{-1}$)</td>
<td>368 [372]</td>
</tr>
<tr>
<td>$\Delta M$ (nm$^{-1}$)</td>
<td>367 [373]</td>
</tr>
<tr>
<td>$\Delta M$ (nm$^{-1}$)</td>
<td>81 [377]</td>
</tr>
<tr>
<td>$\Delta M$ (nm$^{-1}$)</td>
<td>77 [377]</td>
</tr>
</tbody>
</table>

* Determined by steady-state fluorescence quenching according to Eq. (3) by using the binding constants determined by circular dichroism or UV absorption.

The dissociation rate constants were estimated by using the ground-state equilibrium constants, cf. text.

b. Apparent isosbestic points in the UV titration; the second isosbestic point (at higher concentration) is shown in square brackets.

c. Extinction coefficient [at the UV absorption maximum] of the 2:1 complex, extrapolated to quantitative 2:1 complexation.

d. Molar ellipticity [at the maximum wavelength] of the 2:1 complex, extrapolated to quantitative 2:1 complexation.
be more intuitively understood and visualized. The steady-state fluorescence titrations were performed by excitation of the azo group at 367 nm. The concentration of I was held constant at 0.5 mM and the concentration of DMe-β-CD was varied between 0.1 and 20 mM. The fluorescence intensity of the azo group at 367 nm. The concentration of (+)-I-CD was decreased gradually upon addition of DMe-β-CD, see, for example, the change of the emission spectra of (+)-I in the inset of Fig. 5.

The quantitative analysis is based on Scheme 1, which now includes the excited-state complexation equilibria (top). In addition, the following assumptions are made: (1) the dissociation rate constants are sufficiently small to neglect exit of the probe during its excited-state lifetime in the complexes. (2) Differences in radiative decay rates in water and CDs are small [17,43]. These two assumptions are important to derive, for the complexation sequence in Scheme 1, the following analytical expression for the steady-state fluorescence intensity ratio in the presence (I) and absence (I₀) of different CD concentrations:

\[
\frac{I}{I_0} = \frac{R}{S} \times \frac{\tau_0 \cdot \tau_{rel1} + \tau_0 \cdot \tau_{rel2}}{\tau_0 \cdot \tau_{rel1} + \tau_0 \cdot \tau_{rel2}} \times \frac{\tau_{rel1} \cdot [CD] \cdot \tau_{rel2} \cdot [CD]}{\tau_0} \times \frac{\tau_{rel2} \cdot [CD] \cdot \tau_{rel1} \cdot [CD]}{\tau_0} \\
\]  

with

\[
R = \frac{1 + \tau_{rel1} \cdot [CD] + \tau_{rel2} \cdot [CD]}{1 + \tau_{rel1} \cdot [CD] + \tau_{rel2} \cdot [CD]} \\
S = \frac{1 + \tau_{rel1} \cdot [CD] + \tau_{rel2} \cdot [CD]}{1 + \tau_{rel1} \cdot [CD] + \tau_{rel2} \cdot [CD]} \\
\]

Excitation leads to the population of the fluorescent singlet-excited state of the uncomplexed I (I*) with a lifetime τ₀, of the 1:1 complex (CD I*) with a lifetime τ_{CD}, and of the 2:1 complex (CD_2 I*) with a lifetime τ_{CD_2}. The ratio depends directly on the ground-state equilibrium concentrations [I], [CD I], and [CD_2 I] as obtained from Eq. (2) with the known binding constants (Table 1). In addition, the relative extinction coefficients ε_{rel1} and ε_{rel2} need to be considered to correct for differential absorption of the three species in equilibrium (Scheme 1, lower reaction sequence), the excitation wavelength was conveniently adjusted to match the first isosbestic point at 367 nm, where free I and its 1:1 complex absorb equally strong for both enantiomers (ε_{ass1} = 1), while ε_{ass2} was extrapolated from the titration fits in Eq. (1a) and afforded a value of 0.83 ± 0.03 for both enantiomers.

Due to the error statistics in photomultiplier-based fluorescence measurements, which increases with the detected fluorescence intensity (estimated as 3% in the present experiments, reproducibility) the fitting of Eq. (3) to the experimental data was performed in the logarithmic form (Fig. 5) [17]. The difference in fluorescence intensities of the two enantiomers in the region between 2 and 10 mM are experimentally significant, thus presenting an example of chiral recognition through fluorescence quenching [36]. The association rate constants k_{ass1} and k_{ass2} were extracted by a nonlinear least-squares fitting procedure along with the fluorescence lifetimes of the complexes τ_{CD}, and τ_{CD_2} as additional fitting parameters. While the values for k_{ass1}, the rate constants for the formation of the 1:1 complex, were found to be large (>10^7 M⁻¹ s⁻¹) and showed a chiral discrimination (see below), it became immediately evident that the kinetics of formation of the 2:1 complex is too slow to be significant on the time scale of the experiment (ca. 1 μs), such that only a lower limit can be provided (<10^6 M⁻¹ s⁻¹). The ratio de-
Table 1 contains also the fluorescence lifetimes of singlet-excited I in its 1:1 complex (τCD) and in its 2:1 complex (τCD2). The τCD2 values tend to be shorter, which is expected, since the chromophore is fully immersed in the 2:1 complex. However, the fluorescence lifetimes of the complexes are longer than those previously reported for 1:1 complexes of DBO with natural β-CD (10-100 ns) [18]. Presumably, the quenching by DMe-β-CD is somewhat less efficient. It should be noted that there is a chiral discrimination for the fluorescence lifetimes in the 1:1 and 2:1 complexes: τCD was found to be longer, but τCD2 shorter for the (+)-enantiomer. Chiral discrimination on the basis of excited-state lifetimes is known, e.g., for triplet camphorquinone [41].

3.5. Kinetics of formation of the 1:1 complexes and chiral discrimination

The quintessential kinetic parameters extracted from the fluorescence quenching experiments are the association rate constants kass1 for the formation of the 1:1 complexes of both enantiomers of I. First of all, it is important to recognize that the kass1 values for I are larger than for smaller DBO derivatives, and lie in the upper range (>10^9 M^-1 s^-1) of reported association rate constants with CDs in general [18,19,26]. Bohne and co-workers have proposed that larger association rate constants with CDs in general [18,19,26] . The present result obtained for I, a relatively large and more hydrophobic derivative, supports the latter argument. However, differential delocalization effects due to the large structural variation must also be kept in mind [19], since it is the camphane moiety and not the DBO moiety, which is presumably included first (Scheme 1), and therefore determines the kinetics. Finally, one must keep in mind that the presently studied CD is partially methylated, which may enhance the hydrophobicity and association rate constant for this reason, as has been suggested in a previous study [26].

Most important, our data reveal a statistically significant chiral discrimination of the kinetics of CD complex formation, i.e., k(-) > k(+), regardless of whether the binding constants have been taken from either the circular dichroism or UV absorption titrations. While the discrimination is small (ca. 20%), one must keep in mind that previous attempts to manifest such a kinetic discrimination by photophysical measurements have not been successful on account of larger error limits [16]. A large effect was therefore not expected a priori, and in view of the very fast kinetics (close to the diffusion-controlled limit) large differences were also unlikely from a reactivity-selectivity standpoint. But how can there be a significant chiral discrimination on the kinetics, if the variation of the binding constants, on which the analysis of the kinetic data depends upon (see above), is not significant? The first part of the answer is that the binding constants suggest a higher or at best equally high value for (+) as for (-), cf. Table 1. The second part is that the static fluorescence quenching (not considering dynamic quenching by complex formation during the excited-state lifetime) would lead to a larger quenching of the enantiomer which possesses the higher binding constant, i.e., (+), since complexation leads to shorter fluorescence lifetimes. And the third part of the answer is that experimentally, i.e., by including the dynamic fluorescence quenching component, the opposite is observed (more efficient quenching by the (-)-enantiomer), and this deviation can only be rationalized in terms of a faster inclusion of the (-)-enantiomer. This conclusion was confirmed by monitoring the outcome of the fitting even if the extreme error limits of the binding constants were explored. For illustration, we have included in Fig. 5 (dashed lines) the expected static quenching behavior by setting kass1 = 0 in Eq. (4). It is the discrepancy between the dashed and the solid lines for both enantiomers (the different length of the arrows in Fig. 5), which determines the significance of the kinetic chiral discrimination.

The comparison of the thermodynamic and kinetic data in Table 1 reveals that the enantiomer, which tends to show a stronger binding (+), does not show the faster rate constant for complexation, but the slower one. There appears to be no direct correlation between the thermodynamics and kinetics. Similar observations were made in previous studies of CD complexation [18,50], which points to a peculiarity of supramolecular host–guest complexation processes and emphasizes the need for additional case studies to formulate generalized trends in this area.

4. Conclusions

In summary, we have designed the novel bifunctional probe I for chiral discrimination by CDs, consisting of a camphane moiety for chiral information and DBO as a spectroscopic label. DBO can be probed by UV absorption and circular dichroism to obtain information on the microenviron-ment of the chromophore and to determine the binding constants, while the kinetics of association can be obtained from steady-state fluorescence-quenching techniques. An insignif-icant chiral discrimination by DMe-β-CD for the thermodynamics of 1:1 complex formation was observed, favoring the (+)-enantiomer, but a significant one for the kinetics of 1:1 complex formation, now in favor of the (-)-enantiomer. The thermodynamic and kinetic analysis of this host–guest system was complicated by the sequential formation of 1:1 and 2:1 complexes, which led to larger experimental error and more intricate fitting procedures. The application of the new probe to natural β-CD presents a future challenge, since in this case precipitation of the 2:1 complex occurs [38]. Finally, in or-der to obtain more impressive chiral discrimination effects on the kinetics of CD complexation, it appears imperative to choose host–guest systems with association rate constants not approaching the diffusion-controlled limit.
Acknowledgements

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[46] Profil 5.6.3 ed., QuantumSoft, Zürich, Switzerland.
Fluorescence Regeneration as a Signaling Principle for Choline and Carnitine Binding: A Refined Supramolecular Sensor System Based on a Fluorescent Azoalkane**

By Hüseyin Bakirci and Werner M. Nau*

1. Introduction

Water-soluble calixarenes of the p-sulfonato type have numerous envisaged sensor applications for selective recognition of cationic organic guests in aqueous solution.1–14 Owing to its sensitivity and the possibility for optical readout, fluorescence is the technique of choice for many sensor applications.15 However, complexation of fluorescent guests by water-soluble calixarenes has only been studied in selected cases,16 and only a few studies have investigated the effects of analytes on the complexed fluorophores12–14. Moreover, examples of fully water-soluble calixarene–fluorophore sensor systems are scarce.13,14

One promising measurement principle for cation sensing by calixarenes, which has been extensively used for sensing of guests by cyclodextrins (CDs),17,18 involves signaling through a reversible inclusion and competitive displacement of a fluorescent guest with accompanying fluorescence regeneration. This method, also referred to as indicator displacement,19 has been explored with the aim of acetylcholine sensing, first for resorcinarenes,20,21 and later for water-soluble calixarenes.22 p-Sulfonatocalix[4]arene (CX4), the most common derivative, was the best-suited host, owing to its strong binding with acetylcholine.11,12

The original resorcinarene-based sensor operated in alkaline-organic media,20 which was only recently improved by cyano substitution of the host to allow use in neutral aqueous solution.21 A pyrene-appended pyridinium ion has become most popular for use as the fluorescent guest,20,21 but this guest tends to aggregate in water and generally requires the addition of alcohols (1:1). In addition, the fluorescent pyrene group remains exposed to the aqueous bulk (association complex), such that microenvironmental effects on the fluorescence properties are small,20,21 and the observed fluorescence-intensity enhancement in neutral solution remains moderate (1.2–2.7 times).20,21 Alternatively, larger calixarenes than CX4 have been employed as hosts, which apparently allow a deeper inclusion of fluorescent guests (for example, of Rhodamine B13 or dansyl-labeled acetylcholine14), but the use of larger hosts tends to decrease the sensitivity towards external analytes, owing to weaker binding, for example, of acetylcholine, and the fluorescence changes remain small (1.3–2.5 times).

To remedy this situation, we investigate herein the azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) as an alternative fluorescent guest for CX4. The potential of the resulting host–guest complex to serve as a fluorescent sensor for several biochemically important analytes of the choline and carnitine type (see structures which follow) over a large pH range is also assessed. While cholines are well-known for their neurotransmitter function, the carnitines are important for mitochondrial redox metabolism, and are under investigation, among other things, for the treatment of Alzheimer’s dementia. Owing to their structural similarity, carnitines also display a cholinomimetic effect.
2. Results

All experiments described herein were performed in D$_2$O for consistency with $^1$H NMR measurements. The complexation of DBO by CX4 at various pH values was followed by UV spectrophotometry. From the variation of the absorbance with increasing host concentration, the binding constants could be determined by UV titrations and fitting according to a 1:1 complexation stoichiometry (insets in Figs. 1,2); higher-order complexes did not have to be considered. The values were dependent on pH: 4700 ± 200 M$^{-1}$ at pH 2.4, 1200 ± 200 M$^{-1}$ at pH 7.4, and 600 ± 100 M$^{-1}$ at pH 13.2. $^1$H NMR titrations afforded the same binding constants, within error, and provided evidence for the formation of inclusion complexes due to the characteristic complexation-induced up-field shifts (up to 2 ppm).

The precise UV response of the DBO chromophore towards complexation by CX4 warrants special attention. DBO has a weak near-UV absorption of the n,π* type (extinction coefficient, ε = 53 M$^{-1}$cm$^{-1}$, λ$_{max}$ = 364 nm in water), which shows solvatochromic effects that are dependent on the environment, most importantly an increase in its oscillator strength with increasing polarizability/refractive index.[22,23] The characteristic spectral changes have been previously used to determine the polarizability experienced inside the macrocyclic molecules cucurbit[7]uril and CDs.[22–24]

![Figure 1](image1.png)

Figure 1. Variation of the near-UV absorption spectra at pH 7.4 of 2.0 mM DBO (solid line) upon addition of increasing amounts of CX4 in D$_2$O, and corresponding UV titration plot (inset). The dotted line shows the absorption spectrum of protonated DBO at pH 0.5 in the absence of CX4.

At pH values larger than five, the addition of CX4 resulted in a slight bathochromic shift of the DBO UV absorption accompanied by an enhanced oscillator strength (Fig. 1). The oscillator strength, extrapolated to quantitative complexation from the titration plots and corrected for the CX4 absorption, was found to be $f = 10.2 ± 0.2 × 10^{-4}$ at pH 7.4, as well as at pH 13.2. The polarizability ($P$) sensed by the chromophore inside CX4, calculated from the established relationship,[22] amounts to 0.245 ± 0.005. This demonstrates that DBO is expectedly immersed in a more polarizable environment than water ($P = 0.206$), and in one with a polarizability close to dichloromethane ($P = 0.255$) and larger than β-CD ($P = 0.204$).[23] However, the polarizability is lower than in benzene ($P = 0.295$) and much less pronounced than the polarizability extrapolated inside hemicarcerands ($P = 0.45$).[23] Accordingly, the polarizability enhancement inside the calixarenes is relatively moderate, which compares well with the moderately decreased polarity (similar to a 4:1 ethanol/water mixture) estimated inside a water-soluble cationic calix[6]arene, using 1-anilinonaphthalene-8-sulfonate as the solvatochromic polarity probe.[25] Several values of polarizabilities inside supramolecular assemblies are now available; these are listed in Table 1 to provide a current overview.

The UV spectral changes at pH 3.4 and below were different, and have not been previously observed in the supramolecular complexes of DBO.[22,23,26–30] Upon addition of CX4, a decrease in absorbance near 370 nm was observed (Fig. 2), which is in contrast to the expected polarizability-induced increase of the azo absorbance (see above). The depletion of the azo absorption was accompanied by an emerging absorption band near 320 nm (Fig. 2), which was masked as a shoulder in the actual experiment owing to the intrinsic absorption of the CX4 tetramer. On the basis of UV-spectrophotometric control experiments carried out with DBO in strongly acidic aqueous solu-

<table>
<thead>
<tr>
<th>Choline</th>
<th>Acetylcholine</th>
<th>Carbamoylcholine</th>
<th>L-carnitine</th>
<th>O-acetyl-l-carnitine</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH</td>
<td>OCOCH$_3$</td>
<td>OCONH$_2$</td>
<td>CH$_2$CO$_2^-$</td>
<td>OCOCH$_3$</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X</th>
<th>Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH$_2$</td>
<td>COOH</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>COOH</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>COOH</td>
</tr>
</tbody>
</table>

See text for details.

![Figure 2](image2.png)

Figure 2. Variation of the near-UV absorption spectra at pH 2.4 of 2.0 mM DBO (solid line), upon addition of increasing amounts of CX4 in D$_2$O (dashed lines), and corresponding UV titration plot (inset). The dotted line shows the absorption spectrum of protonated DBO at pH 0.5 in the absence of CX4.
tion in the absence of CX4 (Fig. 2, dotted line), this spectral change could be assigned to a partial protonation process, where the hypsochromically shifted absorption band at 320 nm corresponds to the protonated azoalkane. Recall, from a molecular-orbital point of view, that the combination of the two azo nitrogen lone-pair orbitals is elevated upon protonation, thereby increasing the energy gap between the lone pair and the π* orbital, as well as the associated n,π* excitation energy.

The protonation of DBO accounts for the higher binding constant in strongly acidic solution, owing to an additional electrostatic stabilization between cations and the cation receptor, CX4.[7,9,11,31–34]

The complexation process was alternatively monitored through the effect of added CX4 on the characteristic fluorescence of DBO.[35,36] The addition of CX4 caused a fluorescence quenching of this emission (Fig. 3, $k_{\text{exc}} = 365$ nm), which can be rationalized in terms of efficient quenching through hydrogen-atom abstraction from the phenolic hydroxyl groups (cf. $k_q = 9.1 \times 10^8$ M$^{-1}$s$^{-1}$ for p-hydroxybenzene sulfonic acid vs. $k_q = 8.9 \times 10^5$ M$^{-1}$s$^{-1}$ for p-n-propoxybenzene sulfonic acid in D$_2$O, this work), and, possibly, also exciplex formation with the aryl rings.[37–39] The fluorescence quenching of DBO by CX4 is a composite effect, akin to the fluorescence quenching studied for CDs,[26,27] of static quenching in the preformed host–guest complex and dynamic quenching due to diffusive complexation within the very long fluorescence lifetime (505 ns in aerated D$_2$O);[26] in addition, direct dynamic quenching needs to be taken into account.[26] The dissection of static and dynamic fluorescence quenching was not attempted in the present context, since the qualitative observation of efficient fluorescence quenching upon complexation alone paved the way for applications of the CX4-DBO complex as a fluorescent sensor. The stronger (steeper) fluorescence quenching at low pD (2.4) can, however, be readily rationalized in terms of the stronger binding constant (4700 M$^{-1}$ versus 1200 M$^{-1}$).

To demonstrate the use of CX4-DBO complexes in fluorescence-sensor applications, the complex was preformed in D$_2$O in the presence of 1.0 mM DBO and 1.6 mM CX4. Depending on the pD, about 80 % (pD 2.4) or 50 % (pD 7.4) of DBO was complexed under these conditions. The addition of acetylcholine, which is known to bind strongly to CX4,[11] led to a quantitative decomposition of the CX4-DBO inclusion complex, as evidenced by the complete recovery of the UV and $^1$H NMR spectral features of uncomplexed DBO. The release was observed at acidic and neutral pD and is presumed to involve an electrostatic interaction between the positively charged ammonium site and the sulfonato groups of CX4, akin to previously reported complexes with organic ammonium ions.[9,11,31–33,40] This interaction directly affects the binding of (protonated) DBO, thereby leading to the controlled release of the organic guest by competitive binding.

Successive addition of acetylcholine led also to a recovery of the DBO fluorescence ($\lambda_{\text{exc}} = 365$ nm), reaching a plateau at high concentration (Fig. 4). The growth of fluorescence with

![Figure 3](image3.png)

**Figure 3.** Fluorescence quenching of DBO (0.5 mM) by CX4 at pD 2.4 and 7.4; the data were approximately fitted according to a modified Stern–Volmer equation.

<table>
<thead>
<tr>
<th>Host</th>
<th>$P$ [a]</th>
<th>$n$ [b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cucurbit[7]uril</td>
<td>0.12</td>
<td>1.19</td>
</tr>
<tr>
<td>β-CD</td>
<td>0.204</td>
<td>1.33</td>
</tr>
<tr>
<td>DMe-β-CD (2:1 complex)</td>
<td>0.235</td>
<td>1.39</td>
</tr>
<tr>
<td>CX4</td>
<td>0.245</td>
<td>1.41</td>
</tr>
<tr>
<td>Hemicarcerand</td>
<td>0.45</td>
<td>1.86</td>
</tr>
</tbody>
</table>

[a] From the empirical relationship (1/$f = 3020–8320 P$) established in a previous publication.[22] [b] Refractive index, converted using the formula $P = (n^2 − 1)/(n^2 + 2)$.

![Figure 4](image4.png)

**Figure 4.** Variation of the fluorescence spectrum of 1.0 mM DBO in the presence of 1.6 mM CX4 at pD 2.4 upon successive addition of acetylcholine. The inset shows the increase in relative fluorescence intensity at the same DBO/CX4 concentration at pD 2.4 and pD 7.4, fitted according to the competitive binding model in Equation 1.
increasing concentration of additive was fitted by a competitive binding scheme (see Experimental) to afford the binding constants of acetylcholine. Similar fluorescence experiments were performed with additional choline- and carnitine-derived analytes to afford the fluorescence-recovery plots shown in Figure 5 and the combined binding constants listed in Table 2. Binding in alkaline solution was also observed, e.g., a binding constant of ca. 1500 m$^{-1}$ was determined for L-carnitine.

Table 2. Binding constants of choline and carnitine derivatives with CX4, determined by fluorescence regeneration of DBO as a competitively bound fluorescent guest [a].

<table>
<thead>
<tr>
<th>Analyte</th>
<th>$K_C$ ($10^3$ M$^{-1}$)</th>
<th>$pD$ 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N(CH$_3$)$_4$+</td>
<td>2.6 ± 0.1 [25] [b]</td>
<td>12 ± 1 [7.9] [c,d]</td>
</tr>
<tr>
<td>N(CH$_2$CH$_3$)$_4$+</td>
<td>14 ± 1 [4.7] [b]</td>
<td>42 ± 4 [40] [c]</td>
</tr>
<tr>
<td>Choline</td>
<td>1.3 ± 0.1</td>
<td>7 ± 1 [5.0] [c]</td>
</tr>
<tr>
<td>Acetylcholine</td>
<td>1.8 ± 0.1</td>
<td>10 ± 1 [5.0] [c]</td>
</tr>
<tr>
<td>Carbamoylcholine</td>
<td>1.3 ± 0.1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>L-Carnitine</td>
<td>0.63 ± 0.09</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>O-Acetyl-L-carnitine</td>
<td>0.29 ± 0.05</td>
<td>0.14 ± 0.02</td>
</tr>
</tbody>
</table>

[a] In D$_2$O with 1.0 mM DBO and 1.6 mM CX4. [b] Values obtained by microcalorimetric titrations in 0.01 M HCl (pH 2), obtained from a previous publication [33]. [c] Values obtained by $^1$H NMR titrations in 0.1 M phosphate buffer at pH 7.3, from a previous publication [11]. [d] Same value determined in a precious publication [32].

3. Discussion

We have previously introduced DBO as a versatile molecular probe for host–guest complexation phenomena.[22,25–26,30] In addition to being highly water-soluble and uncharged, the molecule is chromophoric and fluorescent, yet, non-aromatic, which differentiates it from most guest molecules previously investigated to afford new insights into the complexation by macrocyclic hosts. DBO has been previously employed to study the kinetics of complexation with CDs by time-resolved fluorescence spectroscopy,[26,27] to study their relative conformation inside CDs by means of induced circular dichroism[28–30] and the polarizability inside cucurbit[7]uril through solvatochromic effects on its UV oscillator strength.[12,22,23] It appeared, therefore, promising to study the complexation of DBO by CX4 with the perspective to estimate the polarizability inside CX4 (see Results) and to advance refined water-soluble fluorescence-based sensors for organic and biomolecular guest molecules, which are in high demand.[11–12] As analytes of prime interest, we have selected choline- and carnitine-derived substrates. The neurotransmitter, acetylcholine, in particular, has an ample and recent history in relation to sensors based on calixarenes[11–14] and related macrocyclic host molecules.[20,21]

As established by UV and $^1$H NMR titrations and fluorescence quenching experiments, DBO forms a 1:1 inclusion complex with CX4 in aqueous solution over a large pH range, with binding constants on the order of 10$^3$ M$^{-1}$. This is an ideal range to allow sizable binding of the fluorescent reporter molecule at millimolar concentrations of host and guest and yet to allow sensitive response to competitive binding, since analytes like acetylcholine are known to bind to CX4 with higher affinity (ca. 10$^5–10^6$ M$^{-1}$).[10]

The addition of CX4 to DBO solutions, i.e., the formation of the inclusion complex, caused a strong fluorescence quenching by about 90% (Fig. 3). The fluorescence quenching of DBO is larger than that observed for the pyrene-appended pyridinium ion by CX4 (ca. 60 %)[20] or resorcinarenes (30–50 %),[21] thereby improving the conditions for efficient fluorescence regeneration by competitive binding of analytes[41]. Indeed, the successive addition of choline and carnitine derivatives, as well as quarternary ammonium ions, resulted in a marked recovery of the DBO fluorescence, which could be accurately followed by measuring steady-state fluorescence spectra and intensities (Figs. 4, 5). The working principle for fluorescence regeneration of DBO from CX4 is simple (Scheme 1): The organic cation displaces DBO (or protonated DBO, at low pH) in the complex, which is therefore released into the aqueous bulk, where static fluorescence quenching by CX4 no longer applies.

As shown in the inset of Figure 4 for acetylcholine, the fluorescence response at pH 2.4 is larger than that at pH 7.4. This is mainly due to the difference in binding constants, which leaves more DBO uncomplexed at neutral pH. The intensity increase, which depends on the actual host/guest concentrations, exceeded a factor of 4 at pH 2.4, which is superior to previous calixarene-based fluorescent sensor systems.[12–14] Note, in particular, that the DBO-based sensor system operates, in principle, over the entire pH range in neat water, i.e., it does not require the use of organic co-solvents and is not restricted to alkaline media like the original resorcinarene system[20] (see Introduction).

The fitting of the fluorescence-recovery curves provided the binding constants of the analytes according to a competitive binding scheme (Table 2). These were generally in good agreement with literature values obtained by $^1$H NMR (values in
The binding constant of the carnitines at pD 2.4 is in- 

tonated at pD 2.4, which should eliminate this charge-repulsion 

based sensors has recently been critically addressed and deeper 

ing of cations at low pH (cf. values for cholines above).

Scheme 1) remains exposed to the aqueous bulk and carries no 

presumably because the substituted group of the guest (R in 

between acetylated, carbamoylated, and unsubstituted choline, 

the experimental data, there is little discrimination by CX4 

related cavitands as macrocyclic hosts.[43] As can be seen from 

the data in Table 2 reveals further that, while the shape 

tion upon competitive binding). The use of DBO provides ac-

cess to a very simple and sensitive, yet non-calorimetric meth-

od for measuring organic-cation binding by CX4 over a large 

range of pH; the method can be employed for the underiva-

ized p-sulfonatocalix[4]arene, but the tethering of DBO as a 

fluorescent guest, akin to the fluorescent-guest-tethered cyclo-
xedtins pioneered by Ueno et al.,[17,18] appears similarly viable, 

since DBO is readily functionalized.[36] The use of DBO as a 

fluorescent guest may be transferable to other water-soluble 

host molecules, such as resorcinarenes or cavatins. For exam-

ple, a recent cavatind study[44] was restricted to 1H NMR deter-

inations, such that the combination of DBO (as a versatile 

fluorescent probe) in combination with deeper cavatins (for 

different and higher selectivity) appears promising.

5. Experimental

CX4 and the organic ammonium salts (as chlorides or hydrochlo-

rides) were purchased from Fluka in highest purity and used as re-

cieved. DBO was synthesized according to a literature procedure [45]. 

All experiments were performed at ambient temperature in D2O 

(99.8 %, AppliChem, Germany). The pD values of the solutions 

were adjusted by the addition of DCI or NaOD. pH readings were taken with 

a WTW 330i pH meter with a combined pH glass electrode (SenTix 

Mic) and then converted to pD (+ 0.40 units) [46]. 1H NMR spectra 

were recorded with a Jeol ECX 400 MHz NMR spectrometer. The 

corrected steady-state fluorescence spectra (λexc = 365 nm) were measured 

with a Varian Cary Eclipse fluorometer. UV spectra were obtained 

using a Varian Cary 4000 spectrophotometer (0.2 nm resolution).

To extract the binding constants of the analytes, or generally any 

competitor, (Kc) from the fluorescence regeneration of DBO, we em-

ployed the accurate mathematical expression for competitive binding 

developed by Wang [47], and adapted it to the competitive binding in 

Scheme . Accordingly, the increase in fluorescence intensity (I) by 

displacement of DBO with varying competitor concentration follows:

\[ \frac{I}{I_0} = 1 + \frac{[\text{CX4}](I_{\text{rel}} - I_{\text{max}})}{K_{\text{DBO}} + [\text{CX4}]} \]  

(1)

where \( I_0 \) is the fluorescence intensity in the absence of competition. 

\( I_{\text{max}} \) is the fluorescence intensity of DBO at quantitative displacement. 

and \( I_{\text{rel}} \) is the relative fluorescence intensity of complexed DBO. \( K_{\text{DBO}} \) is the known binding constant of the complex at the particular pD and 

[\text{CX4}] is the concentration of uncomplexed calix[4]arene. The latter

binding for the carnitines compared to the choline. A similar selectivity pattern was observed 

for the cavatins with carboxylate groups,[44] but not for those with amino groups,[43] which 

reveals the importance of this electrostatic 

design criterion for improving selectivity.

4. Conclusions

The CX4-DBO complex can be employed as a truly water-soluble fluorescent sensor sys-

tem for choline and carnitine derivatives through competitive binding, with concomitant fluorescent guest release. The use of 

DBO as an unconventional fluorophore ensures the formation of a strong inclusion complex as well as fluorescence quenching 

inside the complex (with the desirable fluorescence regeneration 

upon competitive binding). The use of DBO provides access to a very simple and sensitive, yet non-calorimetric method 

for measuring organic-cation binding by CX4 over a large range of pH; the method can be employed for the underivati-

zed p-sulfonatocalix[4]arene, but the tethering of DBO as a fluorescent guest, akin to the fluorescent-guest-tethered cyclo-
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fluorescent probe) in combination with deeper cavatins (for 

different and higher selectivity) appears promising.

square brackets in Table 2). For acetylcholine, binding constants of \( K_C = 1.8 \times 10^4 \text{ M}^{-1} \) at pD 2.4 and \( K_C = 1.0 \times 10^5 \text{ M}^{-1} \) at pD 7.4 were calculated. The higher binding constant of the cholines in neutral solution reflects the enhanced stabilization due to cation–π interactions.[9,31,32,34] A similar trend applies for tetramethylammonium and tetraethylammonium (Table 2). Note that the first pKs of the CX4 phenoxyl groups lies at 3.26 (in H2O),[42] and cation–π interactions are known to be more favorable for the phenolate than for the phenol form, i.e., larger for the CX4 penta-anion dominant in neutral solution.[1,9]
can be derived from the total CX4 concentration, [CX4]₀, by solving the cubic equation:

\[ a[CX4]^3 + b[CX4]^2 + c[CX4] + d = 0 \]  

(2)

where 

\[ a = K_C + K_{DBO} + K_C K_{DBO}([DBO]₀ - [CX4]₀) \]  
\[ b = K_C ([C]₀ - [CX4]₀) + K_{DBO} ([DBO]₀ - [CX4]₀) + 1 \]

The nonlinear fitting of the fluorescence enhancement against the total competitor concentration, [C]₀, was performed with the ProFit software [48]. It provided the pertinent K_C values in Table 2.

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References:
[41] The quenching of DBO by CX4 is also distinct from a photophysical point of view, since it exploits fluorescence quenching through exciplex formation or hydrogen atom abstraction (cf. Results) inside the inclusion complex rather than the most popular polarity response or photoinduced electron transfer, as has been postulated, for example, for the previously employed fluorescent pyrene-appended pyridinium ion [20].
[48] ProFit Version 5.6.3, QuantumSoft, Zürich, Switzerland.
Binding of inorganic cations by \( p \)-sulfonatocalix[4]arene monitored through competitive fluorophore displacement in aqueous solution

Hüseyin Bakirci, Apurba L. Koner and Werner M. Nau*

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A new working principle for detecting inorganic cation binding by water-soluble calix[4]arenes involves the displacement of a fluorescentazoalkane as guest. Fluorescence regeneration is observed for various metal ions, and binding of monovalent cations (alkali and ammonium) to \( p \)-sulfonatocalix[4]arene is detected and quantified for the first time.

Calixarenes are well-established macrocycles with cation receptor properties.1 When substituted with appropriate tethers, calixarenes with astounding selectivity have been reported.2 Calixcrown derivatives soluble in organic solvents have been refined to allow an efficient phase-transfer extraction of cations from aqueous solution, which has led to practical applications as relevant as the removal of radioactive caesium from nuclear waste.3 Highly water-soluble sulfonated calixarenes have become increasingly important,4–18 among which \( p \)-sulfonatocalix[4]arene (CX4) is perhaps the most common derivative. Although its complexion with organic ammonium ions5–7 has been intensively investigated, amongst others due to the potential of acetylcholine sensing,8–10 relatively little work has been expended towards the study of inorganic cation binding,14–17 although sensor systems for metal ions in aqueous solution remain in high demand.19

NMR titrations, which have been broadly employed to study binding of organic ammonium ions with CX4,5–8 are transferable to inorganic cations only in exceptional cases.15 Recently, microcalorimetry has been successfully employed as an alternative to measure the binding constants of several divalent (alkaline earth) and trivalent (lanthanides) cations with CX4;16 this technique is principally suitable also for low binding constants (e.g., 10–20 \( \text{M}^{-1} \)), and in addition yields complexation enthalpies and entropies as thermochemical data, but it is intrinsically unsuitable for thermoneutral, i.e., purely entropy-driven complexations. In contrast, binding of inorganic monovalent cations to CX4 has not been quantified yet and has in fact been neglected in many studies. Moreover, recent microcalorimetric studies have revealed no heat effect upon addition of monovalent cations to CX4, namely ammonium and potassium,14,16 which would be consistent with a negligible binding. On the other hand, early \( ^1\text{H} \) NMR coalescence studies have demonstrated that the presence of alkali ions at pH 8.9 leads to a rigidification of the CX4 cone conformation,4 which implies some form of detectable interaction at least in a qualitative sense and at very high alkali concentrations (0.17–4.7 M).

Herein, we document a sizable affinity of CX4 with monovalent cations, alkali as well as ammonium, which has evaded detection by microcalorimetry, and which has important practical implications, especially when using buffered solutions. The binding constants are on the order of 100 \( \text{M}^{-1} \), i.e., in the same range as for numerous noncharged guests.11–14 We applied a fully water-soluble fluorescence regeneration system recently introduced in the context of acetylcholine sensing.10 It is based on the CX4 complex of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO), an unconventional nonaromatic and uncharged fluorophore (Scheme 1). The system allows for the first time the application of sensitive optical techniques to conveniently study inorganic cation binding with unsubstituted CX4 and to determine their binding constants through a competitive binding scheme,20 sufficiently accurate to expose selectivity trends even in a closely related series of metal ions.

When complexed by CX4, DBO undergoes solvatochromic shifts in the UV absorption spectra, up-field \( ^1\text{H} \) NMR shifts by up to 2 ppm, and strong fluorescence quenching.10 The addition of metal ions led to a decomposition of the CX4-DBO inclusion complex as evidenced by the recovery of the spectral features of uncomplexed DBO (Fig. 1), which was most accurately and very conveniently monitored by steady-state fluorescence.† The release was quantitative for some metals, e.g., La3+, while other metals such as Na+ or Mg2+ displayed smaller effects (Fig. 2). The azoalkane DBO itself is a very poor base21 and ligand22 and does not form bimolecular complexes with the investigated metals in

Scheme 1 Sensor system based on the fluorescence regeneration of DBO by competitive binding of cations with CX4; the lower fluorescence intensity of DBO in the CX4 complex is related to exciplex-induced quenching of singlet-excited DBO by the aryl groups.20

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† Electronic supplementary information (ESI) available: Experimental details. See DOI: 10.1039/b510319c
The corresponding fitting form of CX₄ predominates,²⁶ such that binding of divalent and trivalent cations compare well with the limited thermodynamic trends (all at 298 K and comparable pH). These thermodynamic trends (all at 298 K and comparable pH) are consistent with the expectation, based on relative hydration enthalpies and entropies of the cations, that the decrease in complexity enthalpy with decreasing charge is more pronounced than the corresponding decrease in complexity entropy.¹⁴ A nonvanishing entropic driving force can therefore be rationalized._TRACE_2

Water. The effect of the metal must therefore be ascribed to its complexation by the host. This interaction competes with the binding of DBO, thereby leading to a controlled release of the organic guest by competitive binding according to Scheme 1. An impressive case of metal-ion-induced guest release from calixarenes results, which is documented, among others, for other hosts like cucurbit[6]uril in water²³ and for cavitands in organic solvents.²⁴

The first data set was obtained at pH 2.4, corresponding to pH 2.0 used in previous studies.¹⁵,¹⁶,²⁵ In acidic solution, the tetraanion of 1.6 mM CX₄ in D₂O at pH 2.4.

The contrasting experimental findings, namely that the binding of inorganic monovalent cations is readily detectable by competitive fluorophore displacement but not by microcalorimetry (K° and NH₄⁺)¹⁴,¹⁶ furnishes strong circumstantial evidence for a scarce possibility.¹⁴ With respect to the complexation thermochromism, ΔH° ≥ 0 and ΔTAS ≈ 10 kJ mol⁻¹ results for monovalent cations. For divalent cations, ΔH° ≥ 4 kJ mol⁻¹ and ΔTAS ≈ 23 kJ mol⁻¹ have been reported, and for trivalent ones, ΔH° ≥ 10 kJ mol⁻¹ and ΔTAS ≈ 32 kJ mol⁻¹.¹⁴ These thermodynamic trends (all at 298 K and comparable pH) are consistent with the expectation, based on relative hydration enthalpies and entropies of the cations, that the decrease in complexity enthalpy with decreasing charge is more pronounced than the corresponding decrease in complexity entropy.¹⁴ A thermoneutral complexation of monovalent cations with a nonvanishing entropic driving force can therefore be rationalized.

Fluorescence recovery of DBO can also be monitored in neutral aqueous solution, for which no binding constants of the

---

**Table 1** Ionic radii and binding constants of different inorganic ions with CX₄ in D₂O determined by fluorescence regeneration

<table>
<thead>
<tr>
<th>Ion</th>
<th>Radius/Å²</th>
<th>pD 2.4</th>
<th>pD 7.4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄⁺</td>
<td>1.36</td>
<td>95</td>
<td>165</td>
</tr>
<tr>
<td>Li⁺</td>
<td>0.59</td>
<td>70</td>
<td>80</td>
</tr>
<tr>
<td>Na⁺</td>
<td>1.02</td>
<td>75</td>
<td>85</td>
</tr>
<tr>
<td>K⁺</td>
<td>1.37</td>
<td>100</td>
<td>115</td>
</tr>
<tr>
<td>Rb⁺</td>
<td>1.52</td>
<td>110</td>
<td>135</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>1.67</td>
<td>150</td>
<td>280</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.57</td>
<td>1020</td>
<td>2190</td>
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<tr>
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<td>1590</td>
<td>[1720²][1640]²</td>
</tr>
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<td>1810</td>
<td>4630</td>
</tr>
<tr>
<td>Ba²⁺</td>
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<td>6760</td>
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<tr>
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<td>1.03</td>
<td>23700</td>
<td>52600</td>
</tr>
<tr>
<td>La³⁺</td>
<td>1.03</td>
<td>23700</td>
<td>52600</td>
</tr>
</tbody>
</table>

*¹ 10% error. ² From Handbook of Chemistry and Physics, ed. D. R. Lide, CRC Press, Boca Raton, FL, 84th edn., 2003. ³ By UV absorption titration. ⁴ By ¹H NMR titration. ⁵ Precipitation above 1 mM.

---

**Fig. 1** Variation of (a) the fluorescence spectra and (b) the near-UV absorption spectra for 1.0 mM DBO in the presence of 1.6 mM CX₄ upon successive addition of Ca²⁺ in D₂O at pH 2.4.

**Fig. 2** Increase in fluorescence intensity of 1.0 mM DBO in the presence of 1.6 mM CX₄ illustrating the dependence (a) on cation charge at pH 2.4, and (b) on pH for La³⁺.

**Fig. 3** Increase in fluorescence intensity of 1.0 mM DBO in the presence of 1.6 mM CX₄ illustrating the dependence on cation size (a) for the alkali ions at pH 2.4, and (b) for alkaline earth metal ions at pH 7.4.
investigated cations are known. Unlike the situation in acidic solution, the binding modes of inorganic cations with CX4 in neutral aqueous solution, where one phenoxyl group is deprotonated,26 are not as accurately known. In particular, there is a definitive possibility for complexation at the lower phenoxyl rim,15,17,18 in addition to complexation at the sulphonato rim.6–8,16,25,27 An additional uncertainty arises from the necessary addition of sodium ions (NaOD) to adjust neutral pH, which will affect the observed binding constants (see below). Nevertheless, fluorescence regeneration and competitive binding of the cations was also observed at pH 7.4 (Fig. 2 and 3), and the binding constants resulting from fitting were throughout larger than those in acidic solution (Table 1). This is consistent with an increased binding strength of the cations as a consequence of a higher overall negative charge on the CX4 system and, specifically, with increased cation–π interactions for the CX4 pentaanion.4–7,28

It transpires from the present study that binding of inorganic monovalent cations to CX4 interferes strongly both in acidic and neutral aqueous solution. The presence of residual cations (mostly alkali) with CX4, however, is unavoidable during purification26 and for neutral pH adjustment. While possible adverse effects of buffer constituents have, in fact, remained an immanent issue in complexation studies with CX4,27,29 most studies have implicitly relied on the assumption that the binding of the alkali cations present in the calixarene sample or the cations added to adjust or buffer pH is negligible, which is clearly not the case (Table 1). The accuracy of reported binding constants depends therefore critically on the actual metal ion concentrations present. From simple equilibrium treatment it can be demonstrated that the experimentally observed binding constant of a guest with CX4 (K_{obs}) should fall below the actual value (K_{CX4-Guest}) whenever there is competitive binding with metals (K_{CX4-M}). Eqn. (1) applies for a quantitative release of the guest by an excess of metal ion and defines the systematic error in the measurement. Accordingly, since residual cations in any CX4 sample along with buffer ions typically amount to concentrations of at least 10 mM used in most studies, deviations by a factor of 2 may easily result.

\[ K_{\text{obs}} \approx \frac{K_{\text{CX4-Guest}}}{(1 + K_{\text{CX4-M}}[M^+]_0)} \]  

Since the actual deviation is strongly dependent on the cation content of the sample, and since different buffer solutions have been broadly employed (the use of phosphate buffer concentrations of 0.1 M has not been uncommon),6,8,11,13,30 comparison of literature data must be made with great care. To provide a specific pertinent example, the binding constants for several amino acids (Ala, Leu, Val, Phe, His, Tyr, Trp) titrated at high phosphate buffer concentration (0.1 M)26 were a factor of 20 or more smaller than those measured at lower buffer concentrations,31,32 which can now be accounted for. For the same reasons, thermochemical data determined by microcalorimetry, in particular the complexation entropies, must be used with some caution.

In conclusion, we have introduced a new fluorescence-based method to sensitively monitor and quantify the binding of inorganic cations by CX4 in water at different pH. The method is based on competitive binding and it is noncalorimetric, thereby allowing also the detection of purely entropically driven complexations, as for inorganic monovalent cations. The latter have a sizable binding (ca. 100 M^{-1}) which needs to be considered when comparing binding constants at different metal ion and buffer concentrations.

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Notes and references

Hüseyin Bakirci, Apurba L. Koner, Thomas Schwarzlose, and Werner M. Nau*[a]

Abstract: The pD dependence of the complexation of p-sulfonatocalix[4]arene (CX4) with the azoalkanes 2,3-diazabicyclo[2.2.1]hept-2-ene (1), 2,3-diazabicyclo[2.2.2]oct-2-ene (2), 2,3-diazabicyclo[2.2.3]non-2-ene (3), and 1-methyl-4-isopropyl-2,3-diazabicyclo[2.2.2]oct-2-ene (4) in D_2O has been studied. The pD-dependent binding constants, determined by 1H NMR spectroscopy, were analyzed according to a seven-state model, which included the CX4 tetra- and penta-anions, the protonated and unprotonated forms of the azoalkanes, the corresponding complexes, as well as the complex formed between CX4 and the deuterated hydronium ion. The variation of the UV absorption spectra, namely the hypochromic shift in the near-UV band of the azo chromophore upon protonation, was analyzed according to a four-state model. Measurements by independent methods demonstrated that complexation by CX4 shifts the pK_a values of the guest molecules by around 2 units, thereby establishing a case of host-assisted guest protonation. The pK_a shift can be translated into improved binding (factor of 100) of the protonated guest relative to its unprotonated form as a result of the cation-receptor properties of CX4. The results are discussed in the context of supramolecular catalytic activity and the pK_a shifts induced by different types of macrocyclic hosts are compared.

Keywords: azo compounds · calixarenes · host–guest systems · protonation equilibria · supramolecular chemistry

Introduction

One of the most fascinating aspects of host–guest inclusion complexes is perhaps how the formation of such very simple and discrete supramolecular assemblies is able to modify the chemical reactivity of guests, an important goal in the understanding and mimicking of enzymatic activity.[1,2] One of the simplest ways to alter chemical reactivity is to modify acidity or basicity constants by supramolecular inclusion, which would pave the way for catalytic and biomimetic applications of host–guest complexes and places the focus on the study of water-soluble systems. There are two principal ways to achieve such pK_a shifts, and both are intuitive. The first one involves unspecific hydrophobic interactions resulting from the immersion of an organic water-soluble guest in a nonpolar environment (hydrophobic pocket). This will disfavor ionized sites and states, thereby resulting in reduced and enhanced pK_a values of the ammonium and carboxylic acid groups of amino acids, for example. The second approach requires specific electrostatic interactions with accurate positioning of the acidic or basic functional groups of the complexed guest near regions of negative or positive charge in the host, thereby shifting acidity constants on account of electrostatic repulsions or attractions.

pK_a shifts resulting from unspecific hydrophobic and specific electrostatic effects are well-documented in biological systems,[3–7] and shifts of up to 5 units have been reported, potentially corresponding to a rate enhancement of an acid–base-catalyzed reaction of five orders of magnitude. Although dissection of the various contributing effects is often difficult in enzymes, evidence for predominantly electrostatic effects has been presented in some cases, for example, in acetoacetate decarboxylase.[8] However, relatively little quantitative data have been documented with regard to the pK_a shifts of guests upon binding to water-soluble macrocyclic hosts like cyclodextrins, calixarenes, and cucurbiturils, which could serve as supramolecular models for enzyme–substrate interactions. We have previously observed such pK_a shifts in the complexation of amines by cyclodextrins,[9] and cucurbiturils,[10,11] but without realizing their importance.

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E-mail: w.nau@iu-bremen.de
in a more general context or developing this work towards a systematic understanding of these findings.

We recently observed that \( p \)-sulfonatocalix[4]arene (CX4) shows a pronounced shape complementarity with the non-charged bicyclic azoalkanes 1–4 leading to unexpectedly high binding constants.\(^{[12]}\) It appeared therefore mandatory to investigate to what degree the low basicity of these azoalkanes is affected by the complexation with calixarenes. The purpose of this study was not only to qualitatively invoke such effects on protonation equilibria (which would be trivial because one intuitively expects them), but to analyze them quantitatively, to scrutinize their importance for the complexation mechanism of calixarenes\(^{[13]}\) and to predict their absolute magnitude in terms of the cation-receptor properties of the host. Finally, by extending our previous work on cucurbiturils and cyclodextrins to calixarenes, we expected to be able to establish some general trends for enzyme-mimetic \( pK_a \) shifts in different supramolecular host–guest systems.

The effects on acid–base equilibria induced by tert-butyl-substituted \( p \)-sulfonatocalixarenes were, in fact, noted earlier by Shinkai et al. for the inclusion of large aromatic dyes,\(^{[14]}\) but detailed experimental descriptions and data analyses of the UV spectrophotometric titration data were not provided. In a more recent study, the effect of calixarene complexation on the \( pK_a \) values of stilbene dyes was studied by UV spectrophotometry\(^{[15]}\); in this study, a four-state equation for the pH dependence of binding constants was derived, which, however, was not experimentally tested in terms of pH-dependent binding constants. In this paper, we provide data analyses and experimental tests for a refined seven-state model which previous studies did not focus on.

**Results**

The host-concentration-dependent chemical shifts of azoalkanes 1–4 (see Figure 1 for an example) were employed to determine the complex stoichiometry and binding constants at pH 2.4, 7.4, and 13.2 by \(^1H\) NMR titrations; the consistent formation of 1:1 host–guest complexes was established (Table 1, inset of Figure 2). The binding constants of the azoalkane 2,3-diazabicyclo[2.2.2]oct-2-ene (2) were examined in greater detail over a larger range of pH owing to its importance in recently established fluorescent sensor applications to monitor the competitive binding of choline and carnitine derivatives\(^{[16]}\) as well as inorganic cations\(^{[17]}\) by fluorescence regeneration. The binding constants reported in this work refer to the concentrations of host and guest rather than to their activity. Note, in this context, that a constant ionic strength at varying pH could not be employed because competitive binding of cations\(^{[17]}\) to the host would occur with any added electrolyte.

Bicyclic azoalkanes are very weak bases (\( pK_a = 1.5 \) in \( D_2O \), Table 2) and exist in their unprotonated form in neutral aqueous solution. The binding constants of the unproto-

![Figure 1](https://example.com/figure1.png)

**Figure 1.** \(^1H\) NMR shifts of the \( exo \) and \( endo \) protons of 2 (1.0mM) upon addition of 8 mM CX4 at pH 2.4. The inset shows the \(^1H\) NMR peak-broadening of the methylene protons of CX4 (2.0mM) upon addition of 20 mM 2 at pH 2.4.

<table>
<thead>
<tr>
<th>Azoalkane</th>
<th>pH</th>
<th>( K [m^2] )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2.4</td>
<td>490</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
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</tr>
<tr>
<td></td>
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<td>470</td>
</tr>
<tr>
<td>2</td>
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<td>530</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
<td>1.4</td>
<td>12500</td>
</tr>
<tr>
<td></td>
<td>2.4</td>
<td>4300 [4700](^{[5]})</td>
</tr>
<tr>
<td></td>
<td>3.4</td>
<td>1200</td>
</tr>
<tr>
<td></td>
<td>7.4</td>
<td>900 [1200](^{[5]})</td>
</tr>
<tr>
<td></td>
<td>13.2</td>
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</tr>
<tr>
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<tr>
<td></td>
<td>13.2</td>
<td>580</td>
</tr>
</tbody>
</table>

\(^{[a]}\) The pH was adjusted by addition of DCl or NaOD. \(^{[b]}\) An average value for different protons, as determined by \(^1H\) NMR titration; 10% error. The values at pH 7.4 are from reference [12]. \(^{[c]}\) Determined by UV spectrophotometric titration, taken from reference [16].

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**Table 1.** pH-Dependent binding constants of azoalkanes 1–4 with CX4 in \( D_2O \).

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These are not the final page numbers!
near-UV absorption in water with again at strongly acidic pD values (Figure 2). Binding constants with a maximum at around pD 1.5 characteristic up-and-down feature of the pD-dependent complex with pD in D2O and fitted according to Equation (1). The inset shows the 1H NMR titration plots of the chemical shift of the endo protons at varying pD.

The bicyclic azoalkanes (near pD 7.4) with CX4, which were reported previously,[12] are in the range of around 500–1000 M⁻¹ (Table 1). Interestingly, the binding constants increased by a factor of 5–15 at pD 2.4, except for 1. This increase for azoalkanes 2–4 was attributed to the binding of the protonated azoalkane, that is, the guest is being protonated when complexed to CX4. Azoalkane 1 did not show the same behavior since its pKₐ value is too low (Table 2) to allow protonation in the presence of CX4. Note that the data in Table 1 also reveal that the binding constant of 2 decreased again at strongly acidic pD values (<1). This results in a characteristic up-and-down feature of the pD-dependent binding constants with a maximum at around pD 1.5 (Figure 2).

The bicyclic azoalkanes 1–4 exhibit a characteristic weak near-UV absorption in water with ƛ_{max} (ε) values of 330 (110) for 1, 365 (50) for 2, 377 (70) for 3, and 372 nm (55 m⁻¹·cm⁻¹) for 4. Protonation of azoalkanes results in a diagnostic hypsochromic shift of their UV absorption band (insets of Figure 3).[10,21] The protonation equilibrium of azoalkanes 2–4 in the absence and presence of CX4 was therefore spectrophotometrically monitored to provide information on the acidity constants. The decrease in the near-UV absorbance of uncomplexed 2–4 (in the absence of CX4) due to protonation was followed by recording UV spectra at varying pD. The fitting of this titration according to a two-state model afforded pK_a values in the range of 1–1.5 in D2O (Table 2). Similarly, the decrease in the near-UV absorbance of complexed 2–4 with decreasing pD was followed under conditions of significant (60–90%), pD-dependent) complexation (2mM 2–4, 4mM CX4). As becomes clear from Figure 3, there are substantial differences in the pK_a values of the uncomplexed and complexed azoalkanes. The fitting of the UV titration data in the presence of CX4 was performed according to a four-state complexation model, that is, by considering absorbance contributions from four different forms of the guest (the complexed and uncomplexed and protonated and unprotonated forms, cf. the Experimental Section); this fitting procedure corrects for partial complexation. The resulting acidity constants are listed as pK_a/(UV) values in Table 2. Based on the UV titrations, the pK_a values of the azoalkanes increase by 1.5–2 units upon complexation. Azoalkane 1, however, remained unprotonated down to pD 1 even in the presence of CX4, which prevented the determination of a pK_a shift due to complexation by CX4.

Complexation-induced 1H NMR shifts and 2D ROESY NMR measurements at pD 7.4 have previously afforded evidence for the formation of deep inclusion complexes with an equatorial inclusion geometry for 1–3 and an axial one for 4.[13] The complexation-induced 1H NMR shifts in acidic (Figure 1) and alkaline solutions, determined in this study, are very similar to the data obtained at neutral pD and afforded no indication of a major change in the complexation geometry. An exception was azoalkane 4, for which the bridgehead methyl group showed a significantly larger shift at pD 2.4, which may be indicative of a slightly tilted complex geometry, with the methyl group partially included. Such tilting could improve the centroymmetric electrostatic interaction of the formal positive charge on the protonated azoalkane with the surrounding sulfonato groups (Scheme 1). ROESY spectra obtained for azoalkanes 2–4 at pD 2.4 afforded no significant differences from the spectra at pD 7.4[13] either. We therefore assume that protonation of the azoalkane in acidic solution results in only a minor change in the inclusion geometry from that in neutral solution[13] and propose the complex geometries shown in Scheme 1 for the protonated azoalkanes.

Table 2. pK_a values of azoalkanes 1–4 in their uncomplexed (pK_a) and CX4-complexed state (pK_a') in D2O and extrapolated binding constants of the unprotonated and protonated azoalkanes 1–4 with the CX4 tetra-anion.

<table>
<thead>
<tr>
<th>Azoalkane</th>
<th>pK_a</th>
<th>pK_a' (UV)</th>
<th>pK_a' (NMR)</th>
<th>ΔpK_a</th>
<th>K_{G1}[10^4 mol⁻¹·l⁻¹]</th>
<th>K_{G2}[10^4 mol⁻¹·l⁻¹]</th>
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</thead>
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</table>

[a] Binding constant for the unprotonated azoalkane with the CX4 tetra-anion, cf. Scheme 2. [b] Binding constant for the protonated azoalkane with the CX4 tetra-anion, cf. Scheme 2. [c] Obtained by fitting the experimental binding constants below pD 8 (Table 1) according to Equation (1), see text; 10% error. [d] Estimated from the value of –1.4 in H2O from reference [18] by adding 0.6 units as a typical offset for D2O, cf. reference [19,20]. [e] Taken as the binding constant at pD 7.4, cf. Table 1. [f] This work; the value for 2 in H2O was determined as 0.5, which compares with a value of 0.4 reported in reference [18]. [g] This work; the value of 3.0 for 3 from ref. [18] in H2O appears to be in error. [h] This work.
Discussion

Herein, we describe a quantitative analysis of the pH-dependent binding and host-assisted guest protonation of azoalkanes 1–4, as guests, with p-sulfonatocalix[4]arene (CX4). The first part of the discussion is devoted to the understanding of the accurate complexation mechanism, which was a prerequisite for an accurate determination of the acidity constants. The second part focuses on a generalization and comparison of the observed pK_a shifts of different host and guest molecules.

Mechanism of complexation with CX4: Our interpretations will first concentrate on azoalkane 2. As can be seen from Table 1 and Figure 2, the binding constant of 2 increases from 900 M⁻¹ at pH 7.4 to 12500 M⁻¹ at pH 1.4. This full order of magnitude variation in the binding of an organic guest by CX4 is substantially larger than, for example, the factor of 2–3 observed for amino acids, in which pH-dependent electrostatic effects related to the charged residues cause a minor modulation. As borne out by NMR line-broadening effects, the conformational rigidity of CX4 increases with pH[23,24] as a consequence of different degrees of ionization of the phenoxo groups.[25] The most flexible tetra-anion (with none of the phenoxo groups being deprotonated) converts with a pK_a of 3.26 (H₂O) to the penta-anion, which is transformed with a pK_a of 11.8 (H₂O) to the hexa-anion,[25] this last species is presumed to dominate in strongly alkaline solution,[26] although an additional ionization may occur, judging by the values of pK_a reported for the third and fourth phenoxy ionizations which are subject to larger uncertainty.[23] Nevertheless, CX4 is presumed to adopt a cone conformation across the entire pH range,[23,24,27,28] such that complexation by different conformations appears an unlikely cause of the observed pH-dependent binding affinity. In addition, complexation by the flexible host CX4 is generally presumed to proceed efficiently by an induced-fit mode of action, which in the case of a virtually spherical guest is best met by the postulated conical cavity.[24,29,30] In fact, line-broadening of the methylene peak of CX4 in the presence of an excess of 2 at pH 2.4 (see inset of Figure 1) strongly suggests that the cone shape of the host is stabilized by the presence of the guest even for the most flexible tetra-anion form.[23,24] Note that a pH-dependent change in the complexation geometry of the guest has previously been observed with the trimethylanilinium ion as guest,[23,24,30] but the underlying reasons held responsible, namely differential cation−p interaction, are not relevant to our case. In fact, we have recently demonstrated[28] that cation−π interactions increase the binding at higher pH by a factor of around five as a result of the ionization of the phenolic hydroxy groups of CX4, which produces better electron-donating phenolate aryl sites, yet the observed trends for azoalkanes 2–4 are the opposite.

Surprisingly, although uncomplexed 2 is hardly protonated in D₂O near pH 2.4 (pK_a = 1.0 in D₂O), it is clearly being significantly protonated within the supramolecular complex near pH 2–4, as reflected in the characteristic changes in the UV spectra (insets of Figure 3). This establishes a case for host-assisted guest protonation for calixarenes, which can be directly related to a large pK_a shift. From the UV titrations in the absence and presence of CX4 (Figure 3), the pK_a value for 2 when complexed by CX4 was estimated to be 2.5. The observed protonation of 2 accounts for the en-
hanced binding constant with CX4 at pD 2.4 since, in addition to the hydrophobic interactions,[27,31] there is additional “charge-assisted” binding.[17,30,32] Very similar pKa shifts (1.5–2) were determined for azoalkanes 3 and 4 by UV titrations (Table 2) despite the fact that the pKa value of uncomplexed 3 was shifted by approximately 0.5 units (Table 2) and that the binding constant of 4 at pD 7.4 was about a factor of two smaller than that of 2 (Table 1).

The quantitative understanding of the pD-dependent complexation equilibria in the critical region below pD 8 presented a major challenge. On one hand, CX4 is known to undergo the first ionization of one phenoxy group in this pD range (pKa,CX≈3.9 in D2O, assuming a 0.6 unit offset[19,20] relative to H2O as is commonly found for weak acids).[23] Moreover, the protonation of 2 needs to be taken into account such that at least six species need to be considered. Such a model, however, can only account for an increase in binding at a strongly acidic pD, at which the most stable complex between CX4 and protonated 2 is expected to be formed. Experimentally, however, the binding decreases again at pD values below about 1.4 (Table 1 and Figure 2), such that an additional process needed to be implicated that leads to a destabilization of the complex. Protonation of the sulfonato or phenolic hydroxy groups could be responsible for destabilization of the complex, but this appears unlikely in view of the strongly negative pKa values of arylsulfonic acids[33] as well as protonated phenols (ArOH+).[34] More likely, what is being observed is competitive binding between the deuteriated hydronium ion and the CX4 tetra-anion; such a complexation of inorganic as well as organic cations is expected to lead to competitive binding, a release of 2, and therefore a lower observed binding constant.[16,17]

Consideration of the formation of the hydronium-ion complex of CX4, the existence of which cannot be negated in any case, results then in a seven-state equilibrium (Scheme 2), for which the observed binding constant for complexation between CX4 and 2 can be analytically expressed by Equation (1).

\[
K = \frac{K_{a,G}K_{a,CX}K_{G,2} + K_{a,G}K_{G,1}[H^+] + K_{G,1}K_{H^+}^2}{[K_{a,G} + [H^+]][K_{a,CX} + [H^+] + [H^+]^2/K_{H^+}^2]}
\]  

(1)

As shown in Figure 2, the fitting of data according to the seven-state model in Equation (1) reproduces the experimental results excellently. The thermodynamic parameters obtained from the data fitting are also of great interest. First, it can be seen that the fitted binding constant of unprotonated 2 with the tetra-anion of CX4 (\(K_{G,1}=1000 \pm 200 \text{ m}^{-1}\)) is very similar to that with the CX4 penta-anion (\(K_{G,2}=900 \text{ m}^{-1}\), the value at pD 7.4). The somewhat lower binding in alkaline solution (pD 13.2; \(K_{G,3}=570 \text{ m}^{-1}\)), at which the CX4 hexa- or hepta-anion prevails, can be accounted for by the presence of metal cations (required to adjust the pD), which affect the binding adversely.[17] Importantly, there appears to be no significant increase in binding with increasing cone flexibility (lower pD), which suggests that the differently ionized calixarenes bind the neutral guest with comparable strength. This notion is independently supported by the rather pD-independent binding constants of azoalkane 1 with CX4 (Table 1), since this guest does not undergo protonation as a consequence of its negative pKa value (Table 2). The slightly reduced binding constants for azoalkane 1 in acidic and alkaline solution can again be rationalized by competitive binding by hydronium and alkali ions, respectively.

Secondly, by extrapolation, the binding constant of protonated 2 with the tetra-anion of CX4 (\(K_{G,1}=(1.0 \pm 0.1) \times 10^2 \text{ m}^{-1}\)) was found to be very high, in the upper range of the binding constants of quaternary ammonium ions.[16,23,24,27,35] As can be seen from comparison of the binding constants of unprotonated and protonated 2, the electrostatic contribution to the binding is large (factor of 100), but the hydrophobic effect (which accounts for the binding of the unprotonated form) is larger (factor of 1000) owing to the strong binding of noncharged 2 (Table 2).[12] Thus, our case provides an exception to the general conclusion that electrostatic effects dominate over hydrophobic effects in CX4 binding.[36]

Note in Table 1 that the experimental binding constants for 2 at around pD 1 (ca. 10000 m−1) are nearly one order of magnitude below the extrapolated limit (\(K_{G,1} \approx 1.0 \times 10^5 \text{ m}^{-1}\)) since at this pD binding by the deuteriated hydronium ion has already become strongly competitive. The absolute binding constant for the deuteriated hydronium ion with CX4 (\(K_{H^+,1} \approx 40 \pm 10 \text{ m}^{-1}\)) was found to be very low, however, and corresponds to an apparent “first” pKa value of 1.6 for CX4 in D2O, which is of interest in view of the known difficulties in determining the pKa values of CX4.[23,37,41] The binding constant...
for the hydronium ion is very reasonable in comparison with
the binding constants recently determined for other inorganic
monocations like alkali (70–150 m\(^{-1}\)) and ammonium
(95 m\(^{-1}\)) at pD 2.4.\[17\] In particular, if ammonium has a sizable
binding constant with CX\(_4\), there is absolutely no reason
why the hydronium ion should not also form a complex, and
this mechanistic intricacy is exactly what is required by the
present experimental data. The binding of the hydronium
ion with CX\(_4\) competes, however, only in strongly acidic
media (pD \(<\) 3).

The binding constant of the deuteriated hydronium ion with
CX\(_4\) (obtained for 2) was subsequently kept fixed to estimate
the binding constants for the protonated forms of azoalkanes
3 and 4 with CX\(_4\) as well (two-point fittings from NMR
data!). The resulting values were again very large (Table 2),
in the range of 10\(^5\) m\(^{-1}\). By using the relationship \(K_{GH}/K_{G} = K_{a,G}/K_{a,G}'\) for the pertinent
thermodynamic cycle,\[10\] the pK\(_{a}'\) values for the complex
formed between CX\(_4\) and the protonated azoalkanes could be
independently projected from the NMR data [see the pK\(_{a}'\)-
(NMR) values for azoalkanes 2–4 in Table 2]; these values
were slightly larger than those obtained from the UV spectro-
photometric titrations (Figure 3) which is presumably
related to the use of a four-state model in the latter
method. Conservatively, we have provided the pK\(_{a}'\) shifts
(\(\Delta pK_{a}\) values in Table 2) as an average of the determinations
by the two independent methods with a considerable uncer-
tainty range. The combined data for the different guest mol-
ecules suggest therefore a pK\(_{a}\) shift of around 2 pK\(_{a}\) units
(Table 2).\[42\]

The electrostatic stabilization
of the complex, which is re-
sponsible for the stronger bind-
ing of the protonated form and
therefore the pK\(_{a}\) shift, corre-
sponds to about 10 kJmol\(^{-1}\).
Importantly, this extra stabiliza-
tion is essentially the same as
the total stabilization of the
CX\(_4\) complex with inorganic monocations.\[17\] We therefore
generalize tentatively as follows: The protonation of a non-
charged guest molecule increases the binding with CX\(_4\) by a
factor of around 100; the associated electrostatic stabiliza-
tion adds to an existing hydrophobic stabilization; for small
inorganic cations the binding constants are around 100
m\(^{-1}\) because hydrophobic interactions are absent. It should
therefore be possible to quite reliably predict from the bind-
ing constants of protonated guest molecules those of their
conjugate noncharged forms. This is important, because the

Scheme 2. Mechanism for the complexation of azoalkane 2 with CX\(_4\); data were obtained by using Equa-
tion (1) and refer to equilibria in deuteriated water as solvent (exchanged deuterium atoms are not shown for simplicity).
binding constants of noncharged guests are typically very small and therefore difficult to determine directly experimentally\(^\text{[14]}\) (with the high binding of azoalkanes 1–4 providing a notable exception).\(^\text{[12]}\)

Note that the \(pK_a\) shift reveals an interesting peculiarity of the complexation mechanism between azoalkanes 2–4 and CX4, which is reminiscent of the situation of amine binding by cucurbit[6]uril between pH 10.5–12.\(^\text{[10]}\) In the narrow pH region between around pH 1 and 3, the protonated complex may not only form by direct complexation of the protonated guest, but, alternatively, the unprotonated guest could be preferably complexed owing to its greater abundance in solution. Once captured the basicity of the uncharged guest increases steeply such that rapid protonation occurs to form the more stable protonated guest complex; the net result of the latter mechanism is a complexation accompanied by protonation, that is, a host-assisted protonation (dashed arrow in Scheme 3).

### Complexation-induced \(pK_a\) shifts and catalytic activity

As can be seen, CX4 increases the \(pK_a\) value of bicyclic azoalkanes by around 2 \(pK_a\) units, which is significantly larger than the effect of 1.3 units quantified for the complexation of cyclohexylmethylamine by cucurbit[6]uril\(^\text{[10]}\) and opposite to the situation for cyclodextrins, which were frequently shown to depress the \(pK_a\) value of the conjugate acids of neutral bases by around 0.4–1 units (Scheme 3).\(^\text{[9,43–46]}\) The \(pK_a\) shift for cucurbit[6]uril indicates a positioning of the positive charge in the proximity of the ureidocarbonyl groups, such that stabilizing ion–dipole interactions now favor the protonated ammonium ion over the amine form. The larger \(pK_a\) shift observed for CX4 can also be rationalized since full anionic charges at the sulfonato groups are now involved which allow stronger ion–ion interactions to select the protonated over the unprotonated guest. The inverse \(pK_a\) shift for cyclodextrins is readily accounted for in terms of the relocation of the guest to a hydrophobic environment, which disfavors ionized states in general, and in an un-

The \(pK_a\) shifts of included guests (DBO–4) provide evidence for the selectivity of the water-soluble CX4 have revealed \(pK_a\) shifts of the order of 2, which have been independently determined from the pH-dependent binding constants (by \(^1\)H NMR spectroscopy) and from the pH-dependent changes in the UV absorption spectra of azoalkanes 2–4. The \(pK_a\) shifts result in larger binding constants for the protonated azoalkanes and therefore in an increased binding constant in acidic solution (pH 2.4). In more highly acidic solutions, the binding constant decreases again. This has been attributed to competitive binding by the hydronium ion (\(K\approx40\)) (Scheme 3). The observed host-assisted guest protonation appears to be a rather general phenomenon for macrocyclic cation acceptors and some relationships between the

### Conclusion

In conclusion, the mechanistic investigations into the complexation of azoalkanes 1–4 by the water-soluble CX4 have revealed \(pK_a\) shifts of the order of 2, which have been independently determined from the pH-dependent binding constants (by \(^1\)H NMR spectroscopy) and from the pH-dependent changes in the UV absorption spectra of azoalkanes 2–4. The \(pK_a\) shifts appear to be a rather general phenomenon for macrocyclic cation acceptors and some relationships between the

<table>
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<th>Host</th>
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<th>(pK_a) shift (Cucurbit[6]uril)</th>
<th>(pK_a) shift (CX4)</th>
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<td>4-state</td>
<td>7-state (NMR)/ 4-state (UV)</td>
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\[a\] DBO: 2,3-diazabicyclo[2.2.2]oct-2-ene. \[b\] With protonated guest. \[c\] Induced circular dichroism.

\(\Delta pK_a\) is calculated as the difference between the \(pK_a\) of the free azoalkane and the \(pK_a\) of the complexed azoalkane in water at pH 6.0. The \(\Delta pK_a\) values are determined by \(^1\)H NMR titration method, ICD, and \(^1\)H NMR/UV spectroscopy.

Scheme 3. Water-soluble host–guest complexes exhibiting \(pK_a\) shifts of included guests (DBO = 2,3-diazabicyclo[2.2.2]oct-2-ene).
type of host and guest as well as the interaction topology have emerged. Related $p_K$ shifts are of course well-recognized in biological systems, in which protein-assisted protonation or deprotonation of substrates is of functional importance.[6–7] The design and understanding of supramolecular or polymeric systems that mimic this enzymatic action presents therefore a great challenge[49–51] which should provide an incentive to study additional cation and anion receptors with respect to their ability to modulate the protonation equilibria of organic guests. Finally, host-assisted changes to the ionization states of guest molecules are not limited to $p_K$ shifts but should be transferable to shifts in their redox potentials, the understanding of which is of current interest.[52–55]

Experimental Section

p-Sulfonatocalix[4]arene CX4 ($>97\%$) was purchased from Fluka and used as received. Calixarenes 1–4 were available from previous work.[56] All experiments were performed at ambient temperature in D$_2$O (99.8%, Applichem, Offenbach, Germany). The $pD$ values of the solutions were adjusted by addition of DCl or NaOD. pH readings were taken with a WTW 338 pH meter equipped with a combined pH glass electrode (SenTix Mic) and converted to $pD$ (+0.4 units)[56] where applicable. To obtain $pD$ values, we used 3.2 M DCl and applied an approximate mean activity coefficient of 1.4, based on comparison with tabulated data for HCl.[57] $^{1}H$ NMR spectra were recorded with a JEOL ECX 400 MHz spectrophotometer (0.2 nm resolution) and were corrected with blank measurements in solutions containing only CX4. All experiments were performed at ambient temperature (25°C).

The $pD$ dependence of the binding constants was analyzed by using a seven-state model [Equation (1), see text]; the nonlinear fitting procedure of the ProFit software[54] was employed. The $pD$ dependence of the UV absorbance was approximated using a four-state model (by considering a single protonated and unprotonated complex) as a result of the complexity of the analytical expression already at this level; the model assumes that the guest ($G$) absorbance in the complexes is independent of the degree of protonation of CX4, that is, it is the same for the tetra- and penta-anion complexes.[54] The formula relating to the $pD$ dependence of the UV absorbance was obtained by extension of an expression derived for the fitting of indirect circular dichroism data of protonated versus unprotonated CD–guest complexes.[54] Specifically, the UV absorbance contributions of the uncomplexed protonated and unprotonated guest were included in Equation (2), where A is the experimental UV absorbance normalized for the selected path length ($d$, $\epsilon_{\text{CXG}}$, $\epsilon_{\text{CXGH}}$, $\epsilon_G$ and $\epsilon_{G\text{A}}$ are the extinction coefficients of the unprotonated and protonated complexed and uncomplexed guest at the particular wavelength, respectively, $K_a$ (known) and $K_v$ (to be fitted) are the acidity constants of the uncomplexed and complexed guest, $K_{uq}$ is the apparent binding constant of the unprotonated complex (see Table 2), and $[G]_0$ and $[CX4]_0$ are the total concentrations of guest and host. The fitting of the $pD$ titration data for the free guest was performed according to the usual two-state equation (2).

Acknowledgements

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Host-Assisted Guest Protonation

[42] CX4 also appears to show some selectivity in assisting the protonation of guest molecules, for example, the larger homolog 3 shows a significantly larger pK\(_a\) shift than 2, but the nonspherical derivative 4 displays the same shift as 2.
[58] ProFit 6.0.4, QuantumSoft, Zürich, 2005.
[59] This assumption is justified since the UV absorbance of complexes of 2 with the CX4 penta- and hexa-anions is also the same within error, cf. ref. [16].

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These are not the final page numbers! 
Abstract: Kinetic parameters relevant for the antioxidant activity of the vitamin E constituents (α, β, γ, and δ homologues of tocopherols and tocotrienols) and of an amphiphilic vitamin C derivative, L-ascorbyl 6-palmitate, were determined. Fluorescence quenching experiments of 2,3-diazabicyclo[2.2.2]oct-2-ene (DBO) in homogeneous acetonitrile–water mixtures afforded reactivity trends in terms of intermolecular quenching rate constants, while the quenching of Fluorazophore-L in liposomes provided the lateral diffusion coefficients relevant for understanding their biological activity in membranes. The reactivity in homogeneous solution was not influenced by the nature of the isoprenoid tail (tocopherol versus tocotrienol), but was dependent on the methylation pattern. The resulting order (α > β = γ > δ) was found to be in line with their reactivities toward peroxyl radicals as well as the phenolic O–H bond dissociation energies. The mutual lateral diffusion coefficient in POPC liposomes was the same, within error, for different tocopherols and tocotrienols ($D_L = (1.6 \pm 0.2) \times 10^{-7} \text{cm}^2 \text{s}^{-1}$). L-Ascorbyl 6-palmitate exhibited a reactivity similar to that of δ-tocopherol in homogeneous solution, but displayed a 1 order of magnitude lower fluorescence quenching efficiency in liposomes than the vitamin E constituents. Temperature effects on the laterally diffusion-controlled fluorescence quenching were large, with activation energies of 44±6 kJ mol$^{-1}$. The addition of cholesterol (0–30%) to POPC liposomes resulted only in slightly reduced diffusion coefficients. The combined results demonstrate that Fluorazophore-L can provide important physicochemical parameters for the understanding of antioxidant activity in biological environments.

Introduction

Antioxidants play a vital role in medicine, biology, and polymer chemistry as well as the cosmetics and food industries. Antioxidant activity in lipids, in particular, is of prominent interest for the understanding of lipid peroxidation in membranes$^{1,3}$ and low-density lipoproteins,$^{3-5}$ which is being associated with oxidative stress, a cause of a multitude of diseases,$^{6,7}$ and degradation of food, in particular the rancification of fats.$^8$ Knowledge of the bimolecular reaction kinetics by which antioxidants intercept reactive oxidizing species, as studied herein, is of utmost importance for modeling their actual activity and understanding the mechanisms by which antioxidants act and interact.

Fluorophores based on the azoalkane 2,3-diazabicyclo[2.2.2]-oct-2-ene (DBO), for which we have coined the name fluorazophores,$^9$ display a pronounced radical-like reactivity in their singlet excited state. Owing to their exceedingly long fluorescence lifetimes, they have been introduced as fluorescent probes for antioxidants in homogeneous solution$^{10-13}$ and microheterogeneous systems.$^{14-16}$ The amphiphilic palmitoyl derivative Fluorazophore-L, in particular, has recently been used as a model for reactive membrane-bound radicals to investigate its

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interaction with the water-soluble antioxidant vitamin C at varying pH,15 and with the lipid-soluble antioxidant α-tocopherol.16 These studies have afforded evidence for an unexpectedly enhanced reactivity of vitamin C at low pH, as well as the reaction kinetics in membrane-mimetic lipidic environments.16 The size and type of the lipidic assembly (small anionic versus large neutral micelles versus phospholipid bilayer liposomes) proved to be critical for selecting the appropriate diffusion model. The diffusion coefficient of α-tocopherol in POPC liposomes, which was appropriately analyzed by a lateral diffusion model, could be deduced for the first time from a direct time-resolved measurement,16 thereby correcting previous estimates based on indirect methods.

In fact, the fluorescence quenching of Fluorazophore-L presents a novel approach to study diffusion of membrane additives in real time, which complements alternative approaches based on NMR,17–22 or fluorescence recovery after photobleaching.23–25 The latter two methods are restricted in that they afford only time-averaged information on unreactive membrane constituents or require labeling with usually large aromatic fluorescent probes, thereby modifying the diffusion characteristics to an unknown extent. In the present study, we apply the unconventional fluorescence quenching method for a comprehensive investigation of the diffusive properties of several lipid-soluble antioxidants in liposomes in order to address some standing questions in antioxidant and in particular vitamin E research.

Vitamin E is the most important and effective chain-breaking antioxidant in biological membranes,26–29 although undesirable prooxidant effects have been described under certain circumstances, e.g., in low-density lipoproteins.30,31 One important question is related to the variation in antioxidant activity of the different constituents of vitamin E. Although α-tocopherol is deemed to be the most active compound,32–35 vitamin E is actually a generic term, which includes four tocophersols and four tocotrienols as individual constituents (cf. Chart 1). All of them are potentially active as chain-breaking antioxidants in cellular membranes.2 The chromanol headgroup of tocopherols and tocotrienols is located at the lipid/water phase boundary36–38 and terminates lipid peroxidation by donating a hydrogen atom to a peroxyl radical from its phenolic hydroxyl group.39,40 The isoprenoid side chain is situated in the lipid bilayer and contains, in the case of tocotrienols, isolated trans double bonds at positions 3’, 7’, and 11’ with an E stereochemistry at positions 3’ and 7’.40 Depending on the methyl substitution pattern on the C5 and C7 positions of the chromanol bicycle, α, β, γ, and δ homologues can be further distinguished.41 Not surprisingly, the structural differences between the individual tocopherol and tocotrienol homologues manifest themselves in different in vivo and in vitro activities,42 but to which degree these can be related to differential diffusional properties in membranes has not been scrutinized.

A second question extends to L-ascorbyl 6-palmitate, which is frequently used as an antioxidant additive instead of or in combination with vitamin E. Although this artificial amphiphilic vitamin C derivative is broadly employed to increase the shelf life of foods,43–49 and for cosmetic and pharmaceutical

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Arrhenius plot for the variation of the bimolecular quenching rate constant \( k_q \) in acetonitrile. Shown in the inset is the corresponding Arrehnius plot for the variation of the bimolecular quenching rate constant of (all-rac)-\( \alpha \)-tocopherol versus temperature.

Applications,\(^{50}\) it is not accurately known to which degree it is able to interrupt lipid peroxyl radicals, although it has been suggested to protect and recycle \( \alpha \)-tocopherol in erythrocyte membranes.\(^{51,52}\) Additional open problems are how temperature and the presence of cholesterol, a membrane phase-modulator abnormally found in natural membranes, affect the efficiency by which lipid peroxyl radicals can be intercepted by lipid-soluble antioxidants. This will also be addressed in the present study from the viewpoint of variations of diffusional properties by using fluorescence quenching of Fluorazophore-L as a model reaction.

**Results**

**Quenching in Homogeneous Solution.** For the determination of the bimolecular quenching rate constants, which provide a measure of the hydrogen donor propensity of additives,\(^{10,15}\) we selected degassed acetonitrile–water (9:1) as solvent, in keeping with a previous study.\(^{10,53}\) All investigated antioxidants were found to quench DBO fluorescence efficiently, with close to diffusion-controlled rates, and the quenching plots were strictly linear \((r > 0.99, n = 5, \text{Figure } 1)\). Hydrogen atom abstraction from the phenolic hydroxyl group is the quenching mechanism, as was previously demonstrated through the observation of a deuterium kinetic isotope effect and the tocopheroxyl radical produced in the quenching process.\(^{10,54}\) It should be noted that electron transfer as quenching mechanism can be ruled out on an energetic basis, which has recently been scrutinized for a series of substituted phenols and alkylbenzenes as quenchers.\(^{35}\)

Strikingly, when the phenolic hydroxyl group was acetylated and therefore deactivated (see structure below), fluorescence quenching of DBO became negligible (Figure 1), which provides independent experimental support for the quenching mechanism. Quenching of DBO proceeds thus via hydrogen atom transfer,\(^{54,56,57}\) as does the scavenging of reactive peroxyl or alkoxyl radicals by vitamin E in vitro\(^{38}\) and in vivo.\(^{26,27,59,60}\)

The absolute quenching rate constants were obtained from the slopes of the quenching plots and are listed in Table 1. The value for \( \alpha \)-tocopherol \((k_q \approx 3.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1})\) was consistent with that determined in a previous study.\(^{10}\) The \( \delta \) homologues had the lowest reactivity \((k_q \approx 1.3 \times 10^8 \text{ M}^{-1} \text{ s}^{-1})\), similar to \( L \)-ascorbyl 6-palmitate \((k_q < 1.4 \times 10^8 \text{ M}^{-1} \text{ s}^{-1})\), lowest entry in Table 1). The rate constant of \( L \)-ascorbyl 6-palmitate in the acetonitrile–water mixture was found to be comparable to that of ascorbic acid in water.\(^{15}\) Noteworthy, the rate constant for each tocopherol was the same, within error, as that for the corresponding tocotrienol; that is, the reactivity did not depend on the isoprenoid tail. Moreover, the quenching efficiency of each (all-rac)-tocopherol homologue was the same, within error, as that obtained for the enantiomerically pure \((R.R.R)\) stereoisomer. While this is immediately expected, considering that the reactive site is remote from the chirality centers, one must recall that the different samples had been independently purified.
prepared and purified,\textsuperscript{51–64} such that the similar reactivity of the enantiopure versus all-racemic compounds corroborates sample purity and excludes also that differential impurities are responsible for the observed quenching effects.

The activation energy of bimolecular fluorescence quenching of DBO was determined for α-tocopherol as a representative case. For this purpose, the quenching rate constants were determined in the temperature range from 15 to 50 °C. The resulting Arhennius plot (inset of Figure 1) afforded an activation energy of 10 ± 2 kJ mol\(^{-1}\) in homogeneous acetonitrile—water solution.

**Lateral Diffusion Coefficients in POPC Liposomes.** Fluorazophore-L was included into POPC liposomes, which were prepared by the injection method\textsuperscript{65–67} to allow a homogeneous distribution and accurate adjustment of the concentration of the respective tocopherol or tocotrienol homologue. The fluorescence quenching in liposomes was analyzed according to a lateral diffusion model.\textsuperscript{16} In contrast to fluorescence quenching in homogeneous solution (see above), a time-independent quenching rate constant cannot be defined in this case. Instead, the kinetics of fluorescence quenching in liposomes can be characterized by a mutual lateral diffusion coefficient (\(D_L\)), which can be obtained by global fitting of the decay data according to eq 1.\textsuperscript{16,68} An example of the decays, which were obtained by time-correlated single photon counting, is shown for (R,E,E)-α-tocotrienol in Figure 2.

\[
I(t) = I_0 \exp\left[-(k_d + 2.31D_L N_a [Q_{25}] r + 7.61 \sqrt{D_L R N_a [Q_{25}] r})\right]
\]

The activation energy of bimolecular fluorescence quenching by the tocopherols in the POPC membrane could be extracted from an Arrhennius plot of the mutual lateral diffusion coefficient versus temperature (inset of Figure 2).\textsuperscript{72} The activation energies for α- and δ-tocopherol were found to be 47 ± 5 and 39 ± 4 kJ mol\(^{-1}\), respectively (Table 2).

**Quenching in Cholesterol-Containing Membranes.** Fluorazophore-L quenching by α-tocopherol was also studied in POPC membranes containing cholesterol to better mimic the more heterogeneous composition of biomembranes. Cholesterol is a weak quencher of DBO fluorescence (\(k_q = (2.0 ± 0.2) \times 10^6 \text{molecules cm}^{-2}\) for the employed antioxidants, cf. ref 16), and \([Q_{25}]\) is the two-dimensional concentration of the quencher (antioxidant) on the surface of the liposome. \([Q_{25}]\) ranged generally from ca. 0.8 \(\times 10^{15}\) to 5 \(\times 10^{16}\) molecules/m\(^2\) (corresponding to 0.5–3 mol %) and was calculated by assuming an arrangement with all headgroups oriented toward the aqueous environment. For this purpose, the area per molecule of POPC was taken to be 70 Å\(^2\).\textsuperscript{14} and that of cholesterol to be 38.5 Å\(^2\).\textsuperscript{69} \(I_0\) and \(I(t)\) are the fluorescence intensities at time 0 and \(t\), and \(k_0\) is the unquenched fluorescence decay rate, i.e., the inverse lifetime in the absence of additives (1/\(\tau_0\)). It should be noted that the empirical eq 1 was originally devised to apply to lifetimes around 100 ns.\textsuperscript{16,68} The lifetimes in POPC liposomes (which vary from ca. 70–180 ns depending on temperature and also on cholesterol content, see below) present an ideal match in this respect.

We selected α-tocopherol homologues as the most reactive antioxidants and δ-tocopherol homologues as the least reactive ones for further study in the experimentally highly demanding liposome fluorescence quenching experiments. Their lateral diffusion coefficients (Table 2) were found to be on the order of 10\(^{-7}\) cm\(^2\) s\(^{-1}\), similar to those of lipids.\textsuperscript{70,71} As was found to be the case in homogeneous solution (Table 1), the stereochemistry of the isoprenoid tail has no effect on the lateral diffusion coefficient in the membrane model (entry 1 versus 2 and entry 4 versus 5). Notably, the methylation pattern of the chromanol moiety, which modulates the vitamins’ reactivity in homogeneous solution, does not manifest itself in a sizable difference in the lateral diffusion coefficient (entries 1–3 versus 4–6). Interestingly, no marked influence of the nature of the isoprenoid chain on the diffusion of tocotrienols and tocopherols could be observed either (entry 1 versus 3 and entry 4 versus 6); tocotrienols did not display significantly higher diffusion coefficients than the respective tocopherol homologues.

**Temperature Dependence of Fluorazophore-L Fluorescence Quenching.** The temperature dependence of vitamin E diffusion in POPC liposomes was investigated by determining the mutual lateral diffusion coefficient of the α and δ homologues at five temperatures ranging from 15 to 50 °C (Table 2). The diffusion of the antioxidants increased steeply with temperature, also explaining the significant difference in \(D_L\) between the α-tocopherol value at 25 °C (1.54 \(\times 10^{-7}\) cm\(^2\) s\(^{-1}\), Table 2), the previously reported value at 27 °C (1.8 \(\times 10^{-7}\) cm\(^2\) s\(^{-1}\))\textsuperscript{16} and the value at 30 °C (2.10 \(\times 10^{-7}\) cm\(^2\) s\(^{-1}\), Table 2). The activation energies for fluorescence quenching by the tocopherols in the POPC membrane could be extracted from an Arrhenius plot of the mutual lateral diffusion coefficient versus temperature (inset of Figure 2).\textsuperscript{72} The activation energies for α- and δ-tocopherol were found to be 47 ± 5 and 39 ± 4 kJ mol\(^{-1}\), respectively (Table 2).
10^7 M⁻¹ s⁻¹), similar to alkenes and alcohols,⁷³,⁷⁴ which resulted also in a slight lifetime shortening of Fluorazophore-L in cholesterol-containing liposomes; that is, τₐ dropped from 125 ns to 115, 99, and 81 ns upon incorporation of 5, 15, and 30 mol % cholesterol, respectively. Because the lifetimes remained sufficiently long, i.e., in the recommended range of 100 ns,¹⁶ and because the fluorescence decays remained monoexponential, no additional experimental modifications were necessary, such that the fluorescence quenching in cholesterol-containing POPC liposomes could be studied according to the established protocol. The mutual lateral diffusion coefficients for the probe/α-tocopherol pair were determined at 25 °C for liposomes containing 0, 5, 15, and 30 mol % cholesterol (Table 2, entries 1 and 8–10). Upon incorporation of 5 mol % cholesterol, Dᵥ increased insignificantly. Higher cholesterol contents (15–30%), however, led to a significant decrease, e.g., Dᵥ = 1.36 × 10⁻⁷ cm² s⁻¹ for 30 mol % cholesterol.

### Table 2. Mutual Lateral Diffusion Coefficients and Activation Energies Obtained by Fluorescence Quenching of Fluorazophore-L by Membrane-Bound Antioxidants in POPC Liposomes with Varying Cholesterol Content at Different Temperatures

<table>
<thead>
<tr>
<th>entry</th>
<th>antioxidant type</th>
<th>stereochemistry</th>
<th>cholesterol content/mol %</th>
<th>Dᵥ/(10⁻⁷ cm² s⁻¹)</th>
<th>T/°C</th>
<th>Eₛ/(kJ mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-tocopherol</td>
<td>all-rac</td>
<td>0</td>
<td>0.76</td>
<td>15</td>
<td>47 ± 5</td>
</tr>
<tr>
<td>2</td>
<td>α-tocopherol</td>
<td>R.R.R</td>
<td>0</td>
<td>1.54</td>
<td>25</td>
<td>1.53</td>
</tr>
<tr>
<td>3</td>
<td>α-tocotrienol</td>
<td>R.E.E</td>
<td>0</td>
<td>1.58</td>
<td>30</td>
<td>1.48</td>
</tr>
<tr>
<td>4</td>
<td>δ-tocopherol</td>
<td>all-rac</td>
<td>0</td>
<td>1.48</td>
<td>40</td>
<td>1.36</td>
</tr>
<tr>
<td>5</td>
<td>δ-tocotrienol</td>
<td>R.E.E</td>
<td>0</td>
<td>1.48</td>
<td>50</td>
<td>1.36</td>
</tr>
<tr>
<td>6</td>
<td>ascorbyl 6-palmitate</td>
<td>l</td>
<td>0</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>α-tocopherol</td>
<td>all-rac</td>
<td>5</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>α-tocopherol</td>
<td>all-rac</td>
<td>15</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>α-tocopherol</td>
<td>all-rac</td>
<td>30</td>
<td>0.79</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Error in lateral diffusion coefficient is 10%.*  *b* The data for l-ascorbyl 6-palmitate are apparent values, since the condition of laterally diffusion-controlled quenching may not be fulfilled, cf. text.

systematically dependent on Fluorazophore-L, antioxidant, or cholesterol incorporation.

### Discussion

As set out in the Introduction, knowledge about the fundamental kinetics underlying the oxidative processes in membranes, in particular tocopherol and tocotrienol-mediated radical scavenging, is quintessential for the mechanistic understanding and modeling of the antioxidant action of vitamin E from first principles. The purpose of the present study was therefore to investigate the relative hydrogen donor propensities and the diffusional behavior of lipophilic peroxyl radical-trapping antioxidants in membrane models, both of which are deemed to be important factors in determining antioxidant activity.

#### Structural Effects on Hydrogen Donor Propensity

The strongly fluorescent π,π*-excited state of DBO resembles reactive free radicals in that it is selectively quenched by chain-breaking antioxidants via hydrogen atom transfer.⁵⁴ The exceedingly long-lived fluorescence lifetime of DBO (e.g., 325 ns in aerated water) provides a large time window for collisional encounters, allowing for time-resolved analysis and direct, highly sensitive determination of antioxidant reaction kinetics at physiologically relevant concentrations (µM–mM range).¹⁰–¹⁶ The bimolecular fluorescence quenching rate constant of DBO by an additive provides a measure of its hydrogen donor potency. Indeed, the decrease in rate constant in the order α > β ≈ γ > δ for the tocotrienol and tocopherol homologues determined in the present study (Table 1) resembles that observed for peroxyl radicals,²,³⁴,⁷⁵–⁷⁹ and is consistent with the influence of the chromanol ring methylation pattern on the O–H bond dissociation energy.⁸⁰ The variation in rate constants for Fluorazophore-P (factor 2.3) falls short of that observed for peroxyl radicals (factor 7.3),²⁻³,⁴ which is nicely in line with the reactivity–selectivity principle; that is, the 3rd order of magnitude less reactive peroxyl radicals behave as more selective reactive species. Note that the quenching rate constants of acetylated

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vitamin E constituents (tocotrienyl acetates) were 2 orders of magnitude lower (Table 1), which demonstrates that the isoprenoid tail does not contribute to radical scavenging, but rather the chromanol ring does, with its active hydroxyl group.

The combined data therefore reveal substantial variations in the hydrogen donor propensities for the different vitamin E constituents, but the variation of the isoprenoid tail or stereochemistry has no significant effect (cf. Results). Despite the higher reactivity toward peroxyl radicals of recently reported synthetic vitamin E mimics, remain the most active natural chain-breaking antioxidants known to date, and their high hydrogen donor propensity is once more reflected in the present reactivity data in Table 1.

**Figure 3.** Schematic view of the quenching of Fluorazophore-L in a membrane via lateral diffusion. The long-lived fluorescent singlet excited state produced by excitation of Fluorazophore-L (blue to red) can form an encounter complex with added antioxidant (green), the rate of which is determined by the mutual lateral diffusion coefficient ($D_L$). Once the encounter complex is formed, the antioxidant quenches the fluorescence ($k_d$). The inset shows the presumed transversal immersion patterns of probe and quencher.

The combination of the data therefore reveal substantial variations in the hydrogen donor propensities for the different vitamin E constituents, but the variation of the isoprenoid tail or stereochemistry has no significant effect (cf. Results). Despite the higher reactivity toward peroxyl radicals of recently reported synthetic vitamin E mimics, remain the most active natural chain-breaking antioxidants known to date, and their high hydrogen donor propensity is once more reflected in the present reactivity data in Table 1.

**Structural Effects on Lateral Diffusion.** Differences in the diffusion behavior of the antioxidants in membranes could be studied through a modification of the fluorescence quenching method, which involves Fluorazophore-L as a previously designed amphiphilic DBO derivative. The principle of measurement is depicted in Figure 3: Both the fluorescent probe (blue) and the antioxidant (green) are immersed in a membrane, employing liposomes as models. Upon excitation with a short near-UV laser pulse, the fluorescent probe is promoted to its singlet excited state, which has the important photophysical property of being sufficiently long-lived (70–180 ns, depending on temperature and cholesterol content), to allow diffusional encounter complex formation with an antioxidant molecule. This diffusion occurs in a lateral manner, because the lipid tail is anchored in the hydrophobic region of the lipid leaflet (see inset of Figure 3).

The formation rate of the encounter complex is limited by the mutual lateral diffusion coefficient of the probe/antioxidant pair ($D_L$). Owing to the favorable positioning of the uncharged reactive headgroups of both Fluorazophore-L and the chromanol ring of the vitamin E constituent near the lipid/water interface (see inset of Figure 3), and owing to the high reactivity of the antioxidant, immediate quenching occurs once the encounter complex is formed ($k_d$ in Figure 3), such that the fluorescence quenching reports ultimately on the rate of lateral diffusion. The response of the time-resolved fluorescence decay to varying antioxidant concentrations, which is recorded after the excitation laser pulse, can be employed to extract the mutual lateral diffusion coefficient ($D_L$). It should be emphasized at this point that the liposome size distributions were narrow and monomodal, and no systematic effect of antioxidant addition on the liposome size was noticed (cf. Results). Also important, the custom-made vitamin E constituents had purities far above commercial qualities (generally > 99%, Table 1), such that

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neither differences in sample purity or sizable artifacts due to impurities nor liposome size variations have to be considered further.

Interestingly, when the most reactive (α) and least reactive (δ) vitamin E constituents were immersed in liposomes, they showed, within error, the same efficiency of fluorescence quenching. From a structural (Chart 1) and reactivity (Table 1) point of view, the properties of the β and γ homologues should fall between those of the α and δ ones, such that we suggest comparable rates of lateral diffusion (\(D_L = (1.6 \pm 0.2) \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}\)) for all vitamin E constituents. The fact that the homologues display significantly different reactivity in nonviscous homogeneous solution (Table 1), but exhibit the same efficiency (near an apparently limiting maximum value) of fluorescence quenching when immersed in lipid membranes (Table 2), provides an important experimental manifestation of the diffusion-controlled nature of the quenching process in liposomes. Note that chemical reactions displaying some of the diffusion-controlled nature of the quenching process in nonviscous homogeneous solution (Table 1), but exhibit the same efficiency (near an apparently limiting maximum value) of fluorescence quenching when immersed in lipid membranes (Table 2), provides an important experimental manifestation of the diffusion-controlled nature of the quenching process in liposomes. Note that chemical reactions displaying some selectivity near the diffusion-controlled limit in nonviscous solution (like those of the tocopherols and tocotrienols with DBO in the acetonitrile–water mixtures, Table 1) can easily become diffusion-controlled in somewhat more viscous media, like lipids, such that any remaining differentiation in hydrogen donor propensity is eventually masked.

Intuitively, one expects similar diffusion coefficients for amphiphiles with only one hydrophobic, membrane-anchoring alkyl chain,16 and the same should apply to the tocopherol and tocotrienol homologues, which are structurally even more closely related. It is therefore not surprising that any minor differences in the diffusional behavior become insignificant in liposomes. This means that the addition of methyl groups on the chromanol skeleton does not significantly decrease the diffusion coefficient of the tocopherol (as might be expected from a steric effect), while the change from a saturated hexahydrofarnesyl (or “phytyl”) to an unsaturated farnesyl chain does not result in a significant change in diffusion of the tocotrienols versus tocopherols either. On the basis of this important result, differential diffusion of tocopherols and tocotrienols does not appear to be an important factor in discriminating their antioxidant activity in membranes and organisms.22

Relevance to Radical Scavenging and Antioxidant Activity

With respect to the characterization of the antioxidant potencies of the vitamin E constituents, it is first important to recall (see above) that we have moved from an at least partially reaction-controlled system (fluorescence quenching in nonviscous homogeneous solution, Table 1) to a diffusion-controlled system (fluorescence quenching in the more viscous membrane model, Table 2). This allows one to characterize both the relative hydrogen donor propensity and the diffusion coefficient of the antioxidant by means of two independent experiments. Which of the two factors is more relevant to radical scavenging in membranes depends largely on the reactivity of the reactive free radical that is involved. If free radicals formed in the membrane were highly reactive, akin to the singlet excited state of Fluorozapophor-L, a diffusion-controlled reaction with a chain-breaking lipophilic antioxidant would be expected (unless a faster reaction with the lipid itself competes), such that the efficiency of radical scavenging would be limited by diffusion. If less reactive lipid radicals or oxidizing species are involved, e.g., the biologically most relevant peroxyl radicals, the selectivity will necessarily increase, and the relative efficacy of the antioxidant to donate a hydrogen atom should then become critical.

Second, while the hydrogen donor propensity and diffusion coefficient are jointly of utmost importance to understand and model antioxidant activity in membranes mechanistically and kinetically, the actual biological activity of an antioxidant is dependent on several additional factors affecting its bioavailability, biokinetics, and transport, including resorption, cellular conditions, enzymatic degradation, etc.27,87 For instance, the hepatic α-tocopherol transfer protein selectively incorporates α-tocopherol into membranes and lipoproteins to result in a pronounced plasma preference.88,89 A cytosolic α-tocopherol-binding protein has also been identified, which facilitates incorporation of α-tocopherol into and its transfer between membranes of intracellular organelles.90

In view of the described limitations, any interpretation of the reactivity data in Tables 1 and 2 with previously documented antioxidant effects of different tocopherols and tocotrienols must be made with the necessary caution. For example, the antioxidant potencies of tocopherols and tocotrienols toward peroxyl radicals were shown to increase with the extent of methyl substitution on the chromanol ring system.2,34,75 In contrast, homologous tocopherols and tocotrienols displayed the same antioxidant activity in homogeneous solution,75,81,91,92 in blood plasma,91 in low-density lipoproteins,91 and in liposomes.75 However, other studies in biomembrane models and cellular systems have suggested that tocotrienols are more effective antioxidants than their tocopherol counterparts92–96 and that α-tocotrienol could even provide a better protection against free radical-induced diseases than α-tocopherol,24 for example against lipid peroxidation in supplemented heart tissue.97

The above outlined experimental results are generally in line with the present reactivity data (Table 1) and diffusion coefficients (Table 2), which suggest an enhanced reactivity with increasing methylation, but the same reactivity and diffusional properties for the different isoprenoid chain types. The important contrast that has emerged from the present study is that the lateral diffusion coefficients (Table 2) do not give rise to the expectation of an enhanced antioxidant activity of tocotrienols relative to tocopherols.92–97 Neither does the hydrogen donor propensity differ, nor do the absolute diffusion coefficients suggest a significantly higher mobility of tocotrienols in membranes.


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and co-workers to a higher membrane reorientational dynamics induced by tocotrienol as opposed to a higher ordering effect of tocopherols on the lipidic phase.\textsuperscript{92–93} Close inspection of the results that have led to this conclusion reveals, however, that the corresponding experiments were performed at unnaturally high antioxidant concentrations (20 mol % in DPPC\textsuperscript{92} or 5 mol % in DMPC lipids), such that the implications for lateral diffusion in the context of antioxidant activity remain highly questionable.\textsuperscript{92} For comparison, the present experiments were performed in a concentration range of 0.5–3 mol % vitamin E in POPC lipids, which compares better with the common natural range (0.1 mol % in most organelles\textsuperscript{98} and up to 1.5 mol % in lysosomal membranes of the liver). As exemplified herein for cholesterol (see below and Table 2), additives may well have pronounced effects on membrane properties at high concentration (15–30 mol %), but insignificant ones at lower concentration (≤5 mol %). The effects of α-tocopherol and α-tocotrienol on membrane fluidity may therefore be entirely negligible at physiological concentrations, an opinion that is shared by others.\textsuperscript{75} Moreover, their effects on the membrane surface, where the reactive lipid peroxyl radical sites are presumed to reside, were shown to be essentially the same.\textsuperscript{75} A more “uniform” distribution of α-tocotrienol as compared to α-tocopherol was put forward as an alternative underlying reason for the (putative) higher antioxidant potency,\textsuperscript{93,99} but such reasoning appears to be as much hypothetical as the fluidity effects, and it is not supported by the present fluorescence quenching data. Apart from these in part speculative arguments on the higher antioxidant activity of tocotrienols, one must also keep in mind that most in vitro experiments have not confirmed a higher antioxidant activity of tocotrienols.\textsuperscript{75,81,91,92} In light of the present reactivity and diffusion data, we contend that other, “biological” factors (see above) and not the hydrogen donor propensity and membrane mobility (or related fluidity or uniformity effects) are responsible for the structural dependence of antioxidant activity of tocotrienols versus tocopherols in vivo.\textsuperscript{94–97} For example, recent studies suggest that α-tocotrienol has a higher cellular uptake rate than α-tocopherol.\textsuperscript{95,96}

**Temperature Effects.** We realized that the temperature dependence of fluorescence quenching of Fluorazophore-L in POPC liposomes could provide information on the temperature dependence of the lateral diffusion coefficients and thereby on the activation energies of diffusion in phospholipid membranes. Indeed, dramatic temperature effects on the quenching efficiency of Fluorazophore-L by antioxidants in liposomes were observed, which had several implications. From an experimental point of view, a very precise temperature control with a feedback cycle from a temperature sensor directly positioned in the cuvette was compulsory. From a mechanistic point of view, the large temperature effects implied large activation energies for the diffusion-controlled quenching process, which could be determined by measurements of the lateral diffusion coefficient at different temperatures (Table 2). The resulting Arrhenius plots, which are linear in the investigated temperature range (r > 0.98, n = 5, 15–50 °C, inset of Figure 2), led to activation energies of 47 kJ mol\textsuperscript{−1} for α-tocopherol, 39 kJ mol\textsuperscript{−1} for δ-tocopherol, and 49 kJ mol\textsuperscript{−1} for t-ascorbyl 6-palmitate (see below), all in POPC liposomes (10% error, Table 2). Considering possible sources of uncertainty, we interpret these values conservatively as being the same within error, such that we assign an activation energy of 44 ± 6 kJ mol\textsuperscript{−1} for the fluorescence quenching of Fluorazophore-L by the investigated antioxidants.

The measured activation energy for fluorescence quenching by α-tocopherol in homogeneous solution (10 ± 2 kJ mol\textsuperscript{−1}, in acetonitrile–water, 9:1) was found to be much lower than that in the lipid bilayer system. In terms of formal kinetics, the bimolecular rate constant of a diffusion-controlled reaction depends inversely on the solvent viscosity, such that the experimentally determined activation energies should coincide with the temperature dependence of solvent viscous flow in the different environments. The activation energy in acetonitrile–water lies only marginally above the activation energy for solvent viscous flow in neat acetonitrile (ca. 7 kJ mol\textsuperscript{−1}),\textsuperscript{100} as expected for a close to diffusion-controlled reaction in nonviscous solution. Accordingly, the actual activation energy for fluorescence quenching within the encounter complex should be about 10 kJ mol\textsuperscript{−1} or less. This is consistent with high-level CAS-SCF-calculated activation energies for photoinduced hydrogen abstraction by DBO,\textsuperscript{54,56,57} which constitutes the elementary photoreaction responsible for quenching of fluorazophores by hydrogen donors (k\textsubscript{q} in Figure 3). The fact that the activation energies for Fluorazophore-L quenching in lipid membranes are much higher than this “reaction-controlled” limit, and more close to the activation energies for viscous flow projected from FRAP and NMR studies in POPC or egg-PC membranes (27–41 kJ mol\textsuperscript{−1}),\textsuperscript{24,72,101,102} provides an important piece of experimental evidence that the fluorescence quenching in liposomes is limited by lateral diffusion, and not by the hydrogen transfer step. Jointly with the observed insensitivity toward the hydrogen donor propensity (methylation pattern) of the tocopherol homologues (see above) and the circumstantial evidence reported in our exploratory study (similar positioning in membrane, appropriate fitting according to lateral diffusion model, magnitude of the obtained diffusion coefficients, etc.),\textsuperscript{16} we consider the experimental evidence in favor of a laterally diffusion-controlled quenching of Fluorazophore-L by vitamin E homologues in POPC lipid bilayers to be compelling.

While the small differences between the activation energies determined from the present fluorescence-quenching experiments and those from the previous FRAP and NMR studies may be related to either the investigated diffusing species, the different types of membrane models, or the techniques themselves, the immediate conclusion is as follows: Lateral diffusion of antioxidants increases quite strongly with temperature, even within a small physiological range of 36–40 °C, which may potentially modulate their antioxidant activity.

**Effect of Cholesterol.** To gain more realistic insights into the lateral diffusion kinetics of lipophilic antioxidants, we also studied the effect of cholesterol to better model the situation in natural membranes. We have studied a cholesterol concentration range of 0–30 mol %, to allow comparison with previous diffusion studies by alternative techniques,\textsuperscript{72} and to cover the biologically most relevant concentration range; for example, Golgi apparatus membranes contain approximately 15 mol %.

cholesterol. As can be deduced from the data in Table 2 (entries 1 and 8–10), the diffusion coefficient of α-tocopherol decreases slightly at high cholesterol content to result in a decrease by up to 15%. The data do therefore reveal the expected effect of cholesterol, which causes a rigidification and reduced fluidity or increased microviscosity of the membranes; this limits diffusion of membrane additives, including antioxidants. Accordingly, judging on the basis of diffusional mobility alone, the activity of lipophilic antioxidants could be slightly higher in membranes with lower cholesterol content.

The effect of cholesterol on the mutual diffusion coefficient of the Fluorazophore-L/α-tocopherol probe/quencher pair compares well with the effect reported for the self-diffusion of lipids in the respective macroscopically aligned bilayers by pulsed-field gradient 'H NMR. Close inspection reveals that the decrease in diffusion coefficients is somewhat less pronounced for the fluorescence-based method, which is most likely due to differences in the diffusing species. Thus, while it is commonly accepted that lateral diffusion is slowed by addition of cholesterol, there are also some studies suggesting an enhanced diffusion, e.g., for lecithins, which indicates a differential response of the diffusing species. Filipov et al. have also observed an apparent fluidizing effect at low cholesterol concentrations (2–3 mol %), which was attributed to a reduction in lipid chain entanglements. Also Lemmich et al. reported on a softening of lipid bilayers upon incorporation of small amounts (<3%) of cholesterol. At similarly low concentrations (5 mol %), the diffusion of α-tocopherol in POPC liposomes was in fact not significantly affected (entry 1 versus 8 in Table 2).

The putative patch or raft formation of cholesterol presents another complication, which could lead to either an enhanced or reduced diffusion, depending on whether probe or quencher or both are included in or excluded from the cholesterol patches. The presently applied fluorescence-based technique has distinct advantages, since it is direct and time-resolved and does not imply on time-averaged spectroscopic observations. It may therefore be relevant to mention that our results do not provide evidence for such patch or raft formation; for example, the distribution of fluorescence lifetimes in the absence of antioxidant remains monomodal, and the lateral diffusion model can be identically applied in the presence and absence of cholesterol. Notably, epifluorescence microscopy measurements suggest a domain formation in POPC monolayers only above a concentration of 40 mol % cholesterol. Regardless of those complications and contrasting results, all studies performed on lateral diffusion in cholesterol-containing membrane models, including the present fluorescence quenching work, demonstrate that the addition of cholesterol has no dramatic effects on the mobility of the additives, i.e., their lateral diffusion coefficients. In particular and most important, the cholesterol effect is smaller than that caused by changing the temperature by 2–3 °C (Table 2), such that we rate the presence of cholesterol in natural concentrations as less significant in the context of the rate of diffusion of vitamin E in biomembranes and its consequences for antioxidant activity.

Fluorescence Quenching by l-Ascorbyl 6-Palmitate. l-Ascorbyl 6-palmitate, the lipid-soluble palmitic acid derivative of vitamin C, can penetrate biomembranes, exposing the ascorbyl moiety toward the aqueous environment. It frequently provides a protection against lipid peroxidation, because it is able to protect α-tocopherol from oxidation in lipid environments and to recycle endogenous α-tocopherol radicals during oxidative stress. In contrast to unesterified ascorbic acid, l-ascorbyl 6-palmitate may also scavenge radicals in the core region of low-density lipoprotein particles.

Within the present set of experiments, l-ascorbyl 6-palmitate was an exceptional case since its efficiency of fluorescence quenching in liposomes fell far below the expectations from the quenching rate constants measured in homogeneous solution. Thus, while its reactivity in homogeneous solution is similar to that of δ-tocopherol (Table 1), it becomes more than 1 order of magnitude less reactive in liposomes (Table 2). The immediate conclusion from this contrast, drawn by comparison with the data for the various vitamin E constituents (Table 2), is that the fluorescence quenching of l-ascorbyl 6-palmitate in liposomes is no longer laterally diffusion controlled.

Interestingly, the activation energy for fluorescence quenching is similar to that for the more efficient tocopherols as quenchers (inset of Figure 2), which reveals a major difference in preexponential factors as the underlying reason for the decreased reactivity of l-ascorbyl 6-palmitate. In other words, the reaction is entropically or statistically disfavored compared to tocopherols. We therefore assume that the mutual lateral diffusion coefficient for the Fluorazophore-L/l-ascorbyl 6-palmitate couple is comparable to those of the Fluorazophore-L/vitamin E pairs (cf. activation energies), but that the reaction efficiency upon encounter is very low. This can be rationalized by comparing the charge status. The chromanol headgroup of tocopherols is uncharged near neutral pH, such that a transversal position similar to that of the reactive uncharged headgroup of Fluorazophore-L can be presumed (inset of Figure 3). l-Ascorbyl 6-palmitate has the same alkyl tail as Fluorazophore-L, but its headgroup bears a negative charge (at neutral pH), which unquestionably displaces it more into the aqueous phase, away from the region where the azo chromophore would need to undergo collision-induced quenching and also away from the putative region where peroxy radical groups would float. Such a scenario would require l-ascorbyl 6-palmitate to undergo an up-and-down transversal motion in order to achieve the proper geometry for quenching, which imposes a statistical limit to this reaction within a membrane according to the Collins–Kimball model. A more detailed investigation into the transversal requirements for fluorescence quenching of fluoroazophores will require the design of additional derivatives with shorter and longer alkyl tails, of fluorophore-labeled phos-

photolipids, and the tethering of charged residues, which are subject to future work.

In comparison to the vitamin E constituents studied herein, the present results imply a poorer chain-breaking antioxidant activity of L-ascorbyl 6-palmitate toward reactive lipid radicals as a consequence of a less favorable positioning within membranes. This notion, obtained from fluorescence-based measurements and manifested in an apparently reduced lateral diffusion coefficient, is in fact supported by several independent experimental observations. For example, in membranes containing both α-tocopherol and L-ascorbyl 6-palmitate, it is the tocopherol that is predominantly involved in the primary antioxidant reaction (scavenging of the reactive oxygen species), while the major role of L-ascorbyl 6-palmitate is a recycling of the tocopheroxyl radical product; that is, it is only involved in a secondary process.51,52 Tocopheroxyl radicals have a much longer lifetime than peroxyl radicals, such that a favorable position of L-ascorbyl 6-palmitate is less critical. In fact, the recycling is otherwise known to be performed by water-soluble ascorbate,2,115–118 which statistically resides even less often near the membrane surface.15

Conclusions

Fluorazophores are versatile and convenient fluorescent probes for antioxidants. The bimolecular fluorescence quenching rate constants of antioxidants in homogeneous solution provide a useful measure of their hydrogen donor propensity, which has been established first for glutathione, ascorbic acid, uric acid, and α-tocopherol,10 subsequently for melanin-related metabolites,11 and now for differently methylated tocopherols and tocotrienols. The ease of hydrogen atom abstraction from the phenolic O–H bond increases with increasing methylation degree of the chromanol ring (α > β ≈ γ > δ), and this is reflected in the efficiency of fluorescence quenching in homogeneous solution. In other words, the more active antioxidant (in terms of radical-scavenging reactivity) causes more fluorescence quenching. The synthetic antioxidant L-ascorbyl 6-palmitate is a similarly reactive hydrogen donor as δ-tocopherol in homogeneous solution.

Fluorazophore-L is an amphiphilic derivative of DBO, which can be employed to monitor the interaction with antioxidants near the lipid–water interface. The fluorescence quenching in POPC liposomes as membrane models, which needs to be treated according to the formalism of two-dimensional diffusion and affords a mutual lateral diffusion coefficient instead of a time-independent rate constant, revealed no differences in the quenching efficiency for the different vitamin E constituents. Accordingly, the mutual lateral diffusion coefficient does not vary significantly with the methylation pattern or with the isoprenoid chain type (tocopherol versus tocotrienol). This contrasts with previous suggestions according to which a higher in vivo antioxidant activity of tocotrienols was related, among others, to a membrane fluidizing effect.92,93,119 While the effect of structural variation among the tocopherols and tocotrienols has little effect on the efficiency of fluorescence quenching in POPC liposomes, L-ascorbyl palmitate displayed an unexpectedly low reactivity in liposomes, which is presumably due to its less favorable positioning within the membrane. As a particularly important result, lateral diffusion and therefore fluorescence quenching are strongly affected by temperature, and even a variation of 2–3 °C easily overwhelms the effect of added cholesterol, which decreased the lateral diffusion coefficient by merely 15% even at high concentration (30 mol %).

In summary, Fluorazophore-L allows one to conveniently monitor antioxidants in micro-heterogeneous biomimetic systems in real time by fluorescence spectroscopy and, in limiting cases such as for the vitamin E constituents, to determine the lateral diffusion coefficients of lipid-soluble antioxidants in membrane models. In combination with the fluorescence quenching of DBO in homogeneous solution, which affords relative hydrogen donor propensities, it is therefore possible to routinely and systematically “assay” two complementary kinetic parameters relevant for antioxidant activity.

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Supporting Information Available: Experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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Appendix 2

CURRICULUM VITAE
Curriculum Vitae

Personal Details
Name                  Hüseyin
Surname               Bakirci
Date of birth         25.01.1972
Nationality           French

University Education
1992 – 1993           PCEM1, Faculty of Medicine, University Louis Pasteur, Strasbourg (France).
1996                  B.Sc. in Biochemistry, Major Chemistry, University Louis Pasteur Strasbourg (France).
1997                  M.Sc. in Biochemistry, Major Structural Biology, University Louis Pasteur, Strasbourg (France).
Research Work: "Study of the Importance of Manganese in the Biological Activity of Superoxide Dismutase", under the Supervision of Prof. Dr. Eric Westhof, Institute of Cellular and Molecular Biology.
1998                  M.Sc. in Biochemistry, Major Molecular and Cellular Biology, University Louis Pasteur, Strasbourg (France).
Research Work: "Neurological Disorders Caused by the Oxidative Stress and Apoptosis in the Central Nervous System", under the Supervision of Prof. Dr. Georges Tholey, Institute of Neurochemistry.
1999                  DEA (One-Year Post-Master Degree) in Pharmacology and Medicinal Chemistry, Faculty of Pharmacy, University Louis Pasteur, Strasbourg (France).
Research Project: "Design of New Coupling Strategies of Bacterial Polysaccharides to Preformed Liposomes and Development of Carbohydrate-Based Synthetic Vaccines Against Meningitis", under the Supervision of Prof. Dr. Francis Schuber, Laboratory of Chemical Enzymology and Bioactive Molecules Delivery.
2000 – 2004           Ph.D. in Chemistry with Prof. Dr. Werner M. Nau at the Department of Physical Chemistry, University of Basel (Switzerland).
Curriculum Vitae

2005 Ph.D. in Chemistry with Prof. Dr. Werner M. Nau at the School of Engineering and Science, International University Bremen (Germany).

03 – 04.2006 Post-Doctoral Researcher with Prof. Dr. Jean-Louis Reymond at the Department of Chemistry and Biochemistry, University of Bern (Switzerland).

05.2006 – present Post-Doctoral Researcher with Prof. Dr. med. Hans-Peter Marti at the Division of Nephrology and Hypertension, University Hospital (Inselspital) of Bern (Switzerland).

Teaching Experience


Awards

2003 Third Best Poster Presentation of the National Research Program 47 in Bern (Switzerland).

2006 Elsevier PhD Photoscientist Travel Bursary 2006 for the XXIst Symposium on Photochemistry in Kyoto (Japan).

Attended Lectures during Graduate Studies (University of Basel)

Photochemistry and Photobiology
Computational Chemistry
Investigation of Biological Mechanisms with Spectroscopic Methods
The Molecular and Cellular Basis of Disorders of the Immune System
Deutsch als Fremdsprache