Calixarenes: from biomimetic receptors to multivalent ligands for biomolecular recognition

Francesco Sansone,* Laura Baldini, Alessandro Casnati and Rocco Ungaro

Received (in Montpellier, France) 16th April 2010, Accepted 20th July 2010
DOI: 10.1039/c0nj00285b

Calixarenes are versatile platforms for the design and synthesis of molecular receptors and multivalent ligands that are able to mimic or affect specific biological functions. This review illustrates examples spanning the recognition of small peptides and carbohydrates to ion transport through membranes, biomimetic catalysis, DNA condensation and cell transfection, protein binding, sensing and inhibition, and gives perspectives for using calixarene macrocycles in bio-supramolecular chemistry.

Introduction

Calixarenes[1–5] represent one of the most important classes of macrocycles, together with crown ethers and cyclodextrins. After the initial studies aimed at mastering their conformational properties and the reactivity of their aromatic nuclei (*upper rim*) and phenolic OH groups (*lower rim*), several selective receptors for the inclusion of neutral molecules, metal and ammonium cations and anions based on variably functionalised calixarenes were synthesised. These receptors were used as models to demonstrate theories on weak intermolecular interactions (CH–π, cation–π, H-bonding, *etc.*), for sensing and separation, for catalysis and for many other applications of supramolecular technology. These studies are still continuing with more ambitious and complex goals, such as self-assembly on monolayers[6] and nanoparticles,[7] the formation of rotaxanes and molecular machines,[8] porous materials for gas storage,[9] and dendrimers and systems for molecular encapsulation,[10] certainly making calixarenes protagonists of the *bottom up* approach of nanotechnology.[5]

Along with these applications, in the last ten to fifteen years, researchers involved in calixarene chemistry, we among them, also encouraged by the low toxicity of such macrocycles,[1,11,12] started to tackle more biologically-oriented problems with the aim of investigating the molecular bases that regulate biological processes and of developing new effective therapeutic agents. For these purposes, calixarene derivatives have been used both as biomimetic receptors and as multivalent scaffolds. In the first case, suitably modified macrocycles provide a "minimized" representation of the binding sites of natural receptors, while, in the second, they act as the core of more elaborate branched ligands for biological macromolecules and mimics of portions of cellular surfaces. We can say that a second life has now started for calixarenes, and in this review, which critically accounts for the most relevant results attained up to now by us and other groups, we will try to guide the reader from the birth to the perspectives of this research line.

Dipartimento di Chimica Organica e Industriale, Università degli Studi, Parco Area delle Scienze, 43124 Parma, Italy

Francesco Sansone

Francesco Sansone was born in 1968 in Parma and graduated in chemistry in 1993 from Parma University, spending a period of his undergraduate thesis at Twente University (NL) with Prof. David N. Reinhoudt. In 1995, he developed part of his research on carbohydrates in the group of Prof. Alessandro Dondoni at Ferrara University, and in 1998 he received his PhD in organic chemistry working under the supervision of Prof. Rocco Ungaro. Currently, he is a researcher at Parma University, and his scientific interests are in the field of supramolecular chemistry, in particular, on the synthesis of calixarenes functionalized with amino acids and carbohydrates as biomimetic receptors and ligands for biomacromolecules.

Laura Baldini

Laura Baldini (1974) studied chemistry at the University of Parma and graduated in 1998 from the laboratory of Prof. Rocco Ungaro after developing part of her research thesis at the University of Jyväskylä with Prof. Kari Rissanen. She obtained her PhD with Prof. Rocco Ungaro in 2002 in the field of the self-assembly and molecular recognition properties of functionalized calixarenes. Part of her doctoral studies were carried out at the University of Sheffield (UK) with Prof. Chris A. Hunter. In 2002, she joined the group of Prof. Andrew D. Hamilton at Yale University (USA) as a postdoctoral fellow working in the field of protein surface recognition by synthetic receptors, and since 2005 she has been a researcher at the University of Parma.

In particular, we will focus our attention on calixarene ligands whose biological targets have been clearly or realistically identified. Therefore, we will skip those cases, although interesting, where a generic biological activity has been observed but no data about the mode of binding and the type of interactions have been reported.

Biomimetic recognition, transport and catalysis

Since the birth of supramolecular chemistry, J.-M. Lehn has argued that recognition, translocation and transformation are the three most important functions of supermolecules formed through the non-covalent association of a substrate with its complementary receptor.[13] Within the boundaries of the review outlined in the Introduction, we will discuss in this section only a few selected artificial systems, designed to perform recognition, transport and catalysis with a biological perspective.

Receptors

One of the first examples of bio-inspired molecular receptors based on calixarenes was reported by our group several years ago and refers to the development of biologically active molecules as vancomycin mimics.[14] Vancomycin is a glycopeptide antibiotic, which performs its biological task by binding to the terminal L-Lys–D-Ala–D-Ala sequence of the cell wall mucopeptide precursors of Gram-positive bacteria, thus inhibiting the growth of the cell wall and causing cell lysis. A family of macrobicyclic calixarene derivatives based on model compound **1** was synthesised, having a pseudopeptide bridge in the 1,3-position at the upper rim containing two α-amino acids, AA_1 and AA_2, linked through a 1,3,5-diethylene triamine spacer. NMR diffusion experiments performed in $CDCl_3$ + 3% DMSO-d_6 showed that the calixarene macrocycle having $AA_1 = AA_2 =$ L-Ala binds the N-Ac–Ala–Ala dipeptide, used as model of the bacterial peptidoglycan branch, more strongly (log K_{ass} = 3.4) than the simple amino acid derivative N-Ac–Ala (log K_{ass} = 2.4). Evidence was collected that the carboxylic group of the guest transfers its proton to the amino group of the host, leading to the reasonable conclusion that at physiological pH, the supramolecular complex (**2**) is stabilised by the electrostatic interaction between the charged ammonium and carboxylate groups, supported by hydrogen bonding between the pseudopeptide bridge and the guest. As a matter of fact, some members of the library do really have biological activity very similar to that of vancomycin: they showed an *anti* Gram-positive activity from moderate to good, while no activity was observed toward Gram-negative bacteria (*Escherichia coli*), cell wall-lacking bacteria (*Acholeplasma laidlawii*) or yeasts (*Saccharomyces cerevisiae*).

A topic of current interest in supramolecular chemistry is the design and synthesis of molecules capable of selective recognition of carbohydrates in water, thus called "synthetic lectins"[15,16] or "lectin mimetics".[17] Several strategies have been developed to tackle this difficult task. The most successful is the rational approach reported by A. P. Davis[15] that eventually led to the synthesis of receptors showing selective carbohydrate complexation in water with K_{ass} values very close to the affinity of some natural carbohydrate binding proteins (lectins) for their substrates. This remarkable activity was obtained through the synergy between hydrophobic, CH–π and hydrogen bonding interactions. Carbohydrates are also known to interact with the phosphate anions, and several receptors bearing phosphate or phosphonate groups in their structure have been proposed for the recognition of these substrates.[18,19]

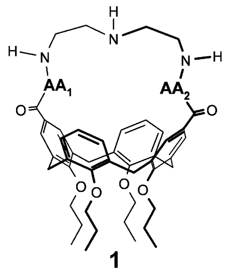
Several of these potential binding elements are incorporated in our "synthetic lectin" prototype **3**:[20] (i) hydrogen bonding donor and acceptor sites are provided by the amino acid units of the bridge; (ii) a charged phosphate group is present in the middle of the pseudopeptide loop; (iii) an apolar cavity is offered by the calix[4]arene in the cone conformation. In addition, receptor **3** possesses four ester groups at the lower rim, which can be used as connecting units to a solid surface or can be hydrolysed to provide a water soluble receptor. Carbohydrate recognition studies by ^1H NMR titration

Alessandro Casnati

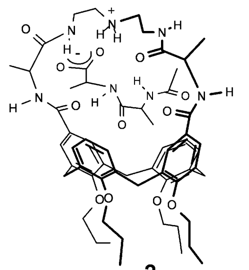
Alessandro Casnati (Milano, 1963) obtained his laurea in chemistry in 1987 and PhD in 1991 from the University of Parma under the supervision of Prof. Rocco Ungaro. In 1990, he spent part of his PhD studies at Twente University (NL) in the laboratory of Prof. David N. Reinhoudt. In 1994, he was appointed a researcher and in 1998 an associate professor of organic chemistry at the University of Parma. In 1997, he was awarded the "G. Ciamician" medal of the Italian Chemical Society. His research interests are in the field of supramolecular chemistry, and particularly in the synthesis and study of molecular receptors for ions and neutral molecules.

Rocco Ungaro

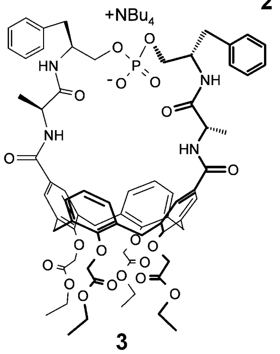
Rocco Ungaro was raised in the South of Italy and graduated in chemistry at the University of Parma in 1968 from the laboratory of Prof. Giuseppe Casnati. In 1974/75 he was a postdoctoral fellow at the State University of New York (SUNY) in Syracuse (NY) in the laboratory of Prof. Johannes Smid. Associate professor in 1982 he became full professor of organic chemistry at the University of Parma in 1986. Prof. Ungaro has pioneered the chemistry of calixarenes and is currently active in the use of these macrocycles in supramolecular and bioinorganic chemistry. In 2002 he was awarded the research prize and in 2009 the Piero Pino gold medal of the Division of Organic Chemistry of the Italian Chemical Society.



1



2



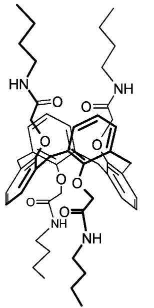
3

experiments were performed in organic media using lipophilic sugar derivatives. The stability constant values in $CDCl_3$ pointed to the good selectivity of this receptor for the β-anomer of octylglucoside with respect both to its α-anomer and to β-octylgalactoside. The association constant dropped dramatically when the anionic phosphate group was transformed into its corresponding acid or methyl ester. The experimental data collected therefore indicate that the phosphate anionic center is the most important binding site, and that additional hydrogen bonds and steric effects determine the observed preference for β-octylglucoside.

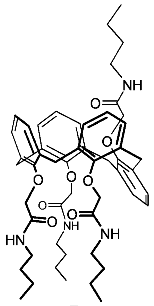
Ion transporters

A further important goal in biomimetic chemistry is the synthesis of receptors that are able to perform substrate transport. Ion transport in nature occurs by means of (a) carriers, (b) pumps and (c) channels.[21] The ion channel function has particularly attracted the attention of many chemists, who have produced a variety of biomimetic systems,[22–24] a few of which are based on calixarenes,[25,26] which are also of biomedical interest since disorder in ion transport is the cause of important diseases. It is well known, for example, that cystic fibrosis is caused by a mutation in the so-called cystic fibrosis transmembrane conductance regulator that controls Cl^- transport.[27] The understanding of Cl^- transfer through membranes and the discovery of new therapeutics are then important goals in this field. The group of J. T. Davis, for instance, obtained interesting synthetic transporters for chloride anion using calix[4]arenes functionalised at the lower rim with four butylamide groups.

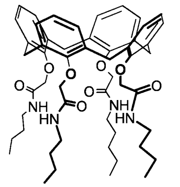
Calix[4]arene derivative **4**, fixed in the 1,3-alternate conformation, once embedded in liposomes, planar lipid bilayers and HEK-293 cells, forms ion channels that move Cl^- across membranes.[28] A single molecule of derivative **4** is too short to span the entire bilayer membrane, but it was proposed that, in the presence of HCl and water, it forms bridged self-assembled species that can act as "aggregate discrete ion channels".[21] The ion transport provided by **4** proceeds either by a H^+/Cl^- symport or a Cl^-/OH^- antiport mechanism. Structural elements are very important in determining the ion transport abilities of this class of compounds. No transport was in fact observed with an acyclic monomeric butylamide and with the less hydrophobic calix[4]arene tetramethylamide, whereas by replacing the secondary amides of **4** with tertiary ones, lacking anion-binding N–H groups, cation transport was detected. Partial cone **5** and cone calix[4]arene derivative **6**[29] both transport Cl^- across phospholipid membranes by a $Cl^-/\ NO_3^-$ antiport mechanism. Evidence, also based on X-ray crystal structures, was collected that, as for calixarene **4**, compound **5** self-assembles to form membrane-active aggregates with ion channel characteristics. Both for **5** and **6**, the introduction at the upper rim of bulky *tert*-butyl groups deactivates the anion transport, probably by inhibiting the formation of the necessary intermolecular aggregates.



4

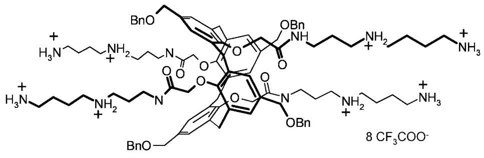


5

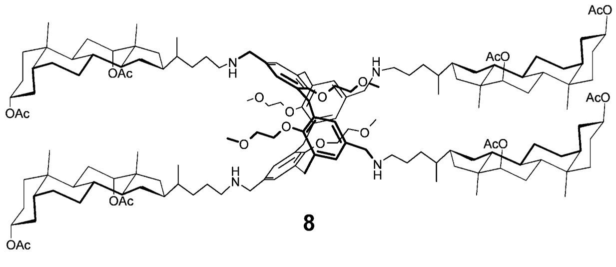


6

A calix[4]arene-spermidine conjugate in the 1,3-alternate conformation (**7**) that, on the contrary, according to molecular mechanics calculations, spans the whole phospholipid bilayer was recently reported by P. Tecilla and F. De Riccardis.[30] It was shown that, when embedded in phospholipid liposomes, this compound is able to selectively transport anions with respect to cations. Iodide and bromide anions are better transported than chloride, while oxo-anions (ClO_4^-, NO_3^-, SO_4^{2-}) are practically not transported. The same authors also reported the H^+ and Na^+ transport activity across vesicle membranes of several conformationally-restricted 1,3-alternate and cone calix[4]arene-cholic acid conjugates.[31] Compound **8**, in the 1,3-alternate conformation, resulted in the most active transporter of Na^+ ions of the series, representing another example of a unimolecular ion channel that spans the entire membrane thickness.

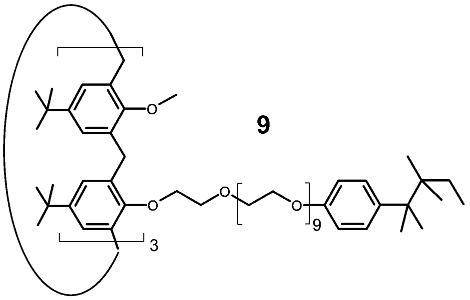


7



8

P. J. Cragg and co-workers, using the knowledge that commercial non-ionic surfactants such as Triton-X-100® destroy liposomes and cell membranes thanks to their ability to penetrate lipid bilayers, decided to harness this quality for the ion specific filtering ability of rigid macrocycles.[32] They synthesized a *p*-*tert*-butyl-calix[6]arene derivative (**9**) having three units of Triton-X-100® in the 1,3,5-positions of the lower rim. Experiments performed on a planar bilayer membrane, which remained intact after the addition of the calixarene conjugate, showed that, in the presence of Na^+, this compound elicits classical ion channel activity. On the contrary, no conductance was observed in the presence of K^+, indicating that the conformational features of the calix[6]arene, together with the presence of six *tert*-butyl groups at the upper and three methoxy groups at the lower rim, allow the formation of a small "hole" through which only Na^+ but not the larger K^+ ion can pass.



9

Catalysts

Enzymes have always fascinated scientists because of their high efficiency and selectivity in catalyzing transformations which are essential for life.[33] Most of the natural and synthetic macrocycles known so far (cyclodextrins, cyclophanes, curcurbiturils, calixarenes, *etc.*) have been used in the last 30 years as platforms for synthesizing enzyme mimics thanks to their ability to form inclusion complexes with several guest species or to organise catalytic groups in space.[33] Among the catalytic systems, a great deal of attention has been devoted to synthetic metallonucleases for the cleavage of phosphodiester bonds in DNA, RNA and their models, which is a more difficult task compared to carboxylic acid ester cleavage.[34]

Our group, in collaboration with those of D. N. Reinhoudt and L. Mandolini, recently synthesized and studied a series of water soluble metallonucleases based on calix[4]arenes and used them for the cleavage of phosphodiester bonds in diribonucleoside monophosphates (NpN′)[35,36] and oligoribonucleotides (from 6 to 17 nucleobases).[37] These catalysts (Fig. 1) are cone calix[4]arenes adorned at the upper rim with one (**10**-Cu), two (**11**-Cu2 and **12**-Cu2) and three (**13**-Cu3) copper complexes of an azamacrocycle ([12]-ane N3).[36]

In the case of NpN′, rate accelerations relative to the background reaction in the order of 10^4 fold were observed, especially for proximal 1,2-dimetallic (**11**-Cu2) and trimetallic (**13**-Cu3) complexes. Comparison with the data obtained using monometallic complexes **14**-Cu and **10**-Cu revealed that two metal centers in complexes **11**-Cu2 and **13**-Cu3 act cooperatively in the cleavage reaction. Interestingly, no metal ion cooperativity was observed in the case of the 1,3-distal complex **12**-Cu2, which behaves essentially as a monometallic complex, and thus reveals a very subtle control of reactivity by the relative position (proximal *vs.* distal) of the two metal centers on the calixarene scaffold. Using **13**-Cu3 as catalyst and 9 out of 16 possible combinations of dinucleosides NpN′, it was found that the most reactive substrates are those having uracyl as the N-nucleobase (UpG and UpU). Taken all together, these results were explained assuming that the uracyl moiety at the 5′-hydroxyl terminus of UpU and UpG is deprotonated and the resulting anion forms a stable copper (II) complex. In this way, one of the copper (II) centers in **11**-Cu2 and **13**-Cu3 acts as an anchoring site for a non-reacting part of the substrate, whereas a second metal center in the 1,2-vicinal position enables the delivery of the internal nucleophile to the metal-activated phosphoryl group. Apparently, due to conformational and geometrical features, this cooperative binding cannot involve metal centers in a 1,3-distal position, thus explaining the low catalytic activity of **12**-Cu2.[36]

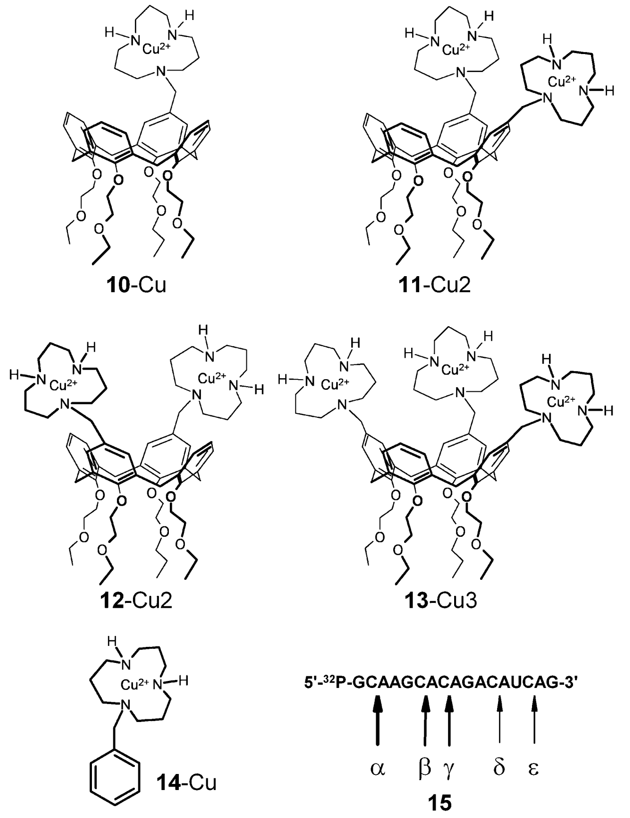


Fig. 1 Calixarene phosphodiesterase mimics, a non-cyclic monomeric model and an eptadecamer used as substrate for hydrolysis reaction (thicker arrows indicate a faster phosphodiester bond cleavage).

Quite interesting results were obtained in the study of the cleavage of oligoribonucleotides of different length, which are closer to the natural RNA.[37] For analytical purposes, all the investigated oligonucleotides contained a terminal radioactive phosphate (^{32}P) introduced in the 5′-terminal position (*e.g.* **15**). Product formation was monitored by gel electrophoresis where only the fragments containing the 5′-end-label were detected. For all metal complexes, a remarkable base selectivity was observed in the cleavage of the CpA phosphodiester bond between cytidine and adenosine nucleosides, in sharp contrast with the UpU and UpG selectivity previously observed in the cleavage of diribonucleoside monophosphates by the same metal complexes. The highest rate acceleration observed in the cleavage of the 5′-pCpA bond was 5 × 10^5-fold, which is one of the highest values recorded so far for the cleavage of phosphodiester bonds in ribonucleotide oligomers. The CpA base selectivity observed closely resembles that shown by natural ribonuclease A and by a number of metal-independent RNase A mimics. The collected data indicate that the modes of interaction of the copper(II) complexes with a diribonucleoside monophosphate and with a 5′-labeled oligoribonucleotide are quite different. In the latter case, the primary interaction between the catalyst and the oligoribonucleotide seems to involve one of the metal centers and the ^{32}P-radiolabeled 5′-terminal phosphate dianion, and the subsequent cleavage occurs at the intrinsically most reactive CpA phosphodiester bond. An important observation that supports this interpretation was made in the cleavage of heptadecamer **15** in the presence of both dimetallic complexes **11**-Cu2 and **12**-Cu2, which, in this case, showed similar reactivity and selectivity. Substrate **15** features six pyrimidine-pN sequences, five of them being CpA, which were all cleaved, whereas other bonds did not undergo appreciable scission in more than 6 h. CpA bonds labelled with α, β, and γ, which were closer to the phosphate group in the 5′-terminal position, were cleaved much more rapidly than those labelled δ or ε, which were far away.[37] The main conclusion of this study was that for oligoribonucleotide or RNA substrates having a terminal double-charged phosphate group, the catalyst–phosphate electrostatic interaction, rather than base complexation, may become the dominant factor in determining the reactivity and selectivity of phosphodiester bond cleavage.

Multivalent ligands for the binding to natural macromolecules

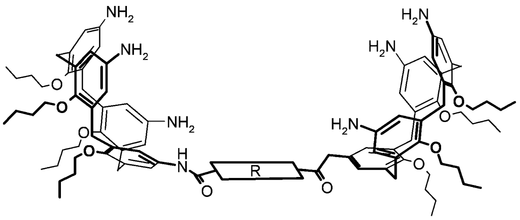
In the examples reported above, the substituents linked to the calixarene structure work as fingers that converge towards an object, the substrate, to catch it. On the other hand, when suitable recognition sites are present, these functionalities can be seen as fingers that slide into the holes of a bowling ball. In this case, the calixarene gains the role of scaffold, directing its ligand units to the identical binding sites of the target, which can be either a multiple binding site receptor or a self-assembled system.

Many biological processes are characterized by a phenomenon called multivalency,[38] which consists of the simultaneous interaction between several identical units of one partner and the corresponding recognition sites of the other. Thanks to multivalency, two entities (or molecules) interact with an affinity and specificity that is often higher than the sum of the single monovalent interactions.[39,40] Therefore, the versatility of calixarenes as multivalent scaffolds, together with the important role played by multivalency in nature, puts these macrocycles stably in harness with the biological world, as the following examples will show.

DNA binding and cell transfection

In spite of the relevance of nucleic acids for life and of the undoubted appeal for DNA/RNA targeting or mimicking, researchers active in calixarene chemistry have not paid much attention to these natural macromolecules within the context of bio-supramolecular chemistry until very recently.

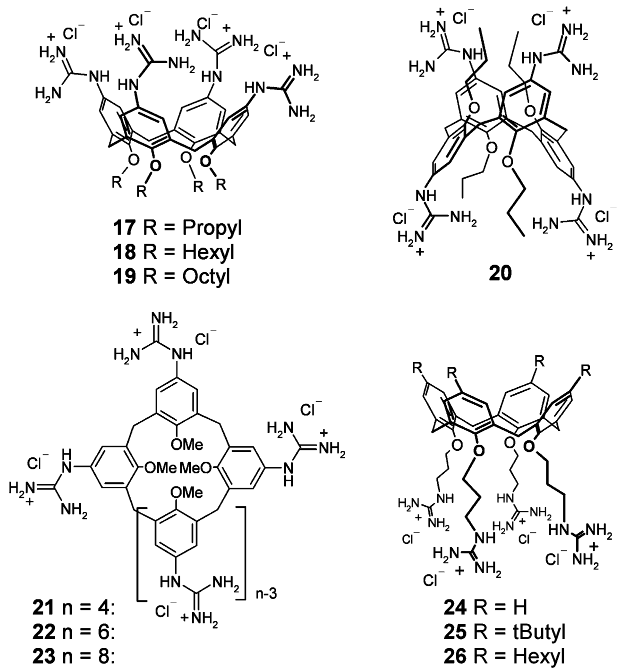
After the first interesting results reported by H.-J. Schneider at the end of the 1990s,[41] only several years later did R. Zadmard and T. Schrader describe the synthesis of two calixarene dimers[42] (**16**) with a total number of six primary amines at the upper rim that are able to bind dsDNAs and RNAs of different length. These compounds show K_{ass} values in the 10^5–10^7 M^{-1} range and, apparently, do not cause conformational changes or destabilization in the duplex. Preference for RNA over DNA, and for polyGC over polyAT, suggested that the interaction is probably assisted by the insertion of a portion of the ligand into the major groove of the double helix, and that it does not simply occur through the formation of a multiple salt bridge.



16 R = $-(CH_2)_4-$ or Ph

More recently, the interaction with DNA of a tetraamino-calix[4]arene having long dodecyl chains at the lower rim was investigated by P. Shahgaldian and co-workers.[43] This amphiphilic compound forms monolayer Langmuir films at the air–water interface whose area increases by increasing the DNA concentration in the aqueous solution as a consequence of the host–guest interaction. The same derivative self-assembles into nanoparticles when dispersed in water, and evidence of its ability to bind DNA also in this form was preliminarily reported.

Our main interest in this field is focused on the use of cationic calixarenes for gene delivery and cell transfection. When we started this work, no examples of calixarenes performing these important functions were present in literature. Therefore, we initially synthesized calix[*n*]arenes **17**, **19**, **22** and **23** functionalized with guanidinium groups at the upper rim and reported their preliminary DNA binding properties.[44] We chose this charged group, rather than the amino function, because it is protonated over a wide pH range and because it has a specific and strong binding towards phosphate anions. Widely-spread in biological environments, guanidinium is also frequently used in supramolecular chemistry for anion recognition. We proved that these compounds are able to bind plasmid DNA through electrophoresis mobility shift assays (EMSA) and, importantly, no relevant toxicity was revealed through detection of the metabolic activity of treated cells. These preliminary data encouraged us to pursue a more systematic approach for developing macrocyclic vectors based on calixarenes, and we synthesized a small library of potential vectors with different conformational properties, sizes and lipophilicity/hydrophilicity ratios[45] (Fig. 2).



17 R = Propyl
18 R = Hexyl
19 R = Octyl

20

21 n = 4:
22 n = 6:
23 n = 8:

24 R = H
25 R = tButyl
26 R = Hexyl

Fig. 2 Upper and lower rim guanidinocalixarenes studied as non-viral vectors.

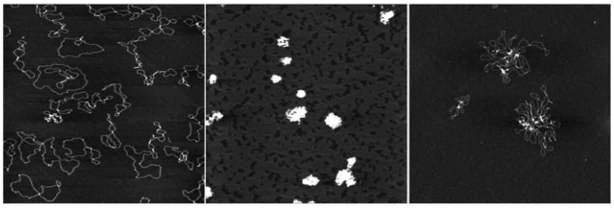


Fig. 3 AFM images in tapping mode on air (2×2 μm scans) of pEGFP-C1 plasmid DNA 0.5 nM alone (left), + cone tetraguanidinocalix[4]arene **17** 1 μM (center), and + guanidinocalix[8]arene **23** 1 μM (right).

Although the data must be considered as only qualitative, gel electrophoresis, ethidium bromide displacement assays and melting curve determinations all agreed in evidencing substantial differences between the ligands in their ability to interact with DNA, which became clearer after atomic force microscopy (AFM) experiments. The derivatives in the cone conformation (**17–19**) are able to condense DNA filaments (Fig. 3, center) through the combined action of charge–charge interactions between guanidinium and phosphate groups, followed by a collapse of the complex induced by additional hydrophobic interactions between the aliphatic tails at the lower rim of the ligands. This condensation process can also justify the double helix stabilization we observed for these ligands in the melting curve determination and also previously revealed by Schneider[41] for cone permethyl-ammoniumcalixarenes. 1,3-Alternate **20** and mobile calixarenes **21–23**, on the contrary, act as binders only through charge–charge interactions. Moreover, calix[4]-arenes **20** and **21** condense DNA, while the larger calix[6]arene **22** and calix[8]arene **23** form disordered multiplasmid aggregates (Fig. 3, right). In agreement with this behaviour, as revealed by AFM, guanidinocalixarenes that are not able to form nanometric condensates do not give transfection, while the others deliver DNA into the cells, either alone or in the presence of the dioleoylphosphatidylethanolamine (DOPE) helper lipid. The use of this adjuvant is necessary for derivatives lacking a significant lipophilic portion, such as **17**, while it is not required for tetrahexyl and tetraoctyl compounds **18** and **19**. Importantly, from these data, we found a relationship between the biological activity, the mode of binding to DNA, and the structural and conformational properties of the ligands. However, a relatively high cytotoxicity and a transfection efficiency that does not exceed the 20% of the treated cells (Fig. 4) characterize the activity of this first generation of gene vectors based on calixarene scaffolds towards Rhabdomiosarcoma cells. This cell line was chosen for our experiments because it is medically relevant, easy to grow and not very easily transfectable by traditional methods.

Our subsequent efforts to improve transfection efficiency and reduce toxicity were successful when we moved the guanidinium units from the upper to the lower rim[46] (Fig. 2). In particular, compound **24** is a very good vector for gene delivery in presence of DOPE, with a low toxicity and a higher transfection efficiency than the upper rim guanidinocalixarenes and, more importantly, significantly higher than a commercially available lipofectamine (LTX) formulation towards Rhabdomiosarcoma (Fig. 4) and Vero cells. Instead, the presence of the alkyl chains at the upper rim, as in ligands **25** and **26**, increases the toxicity, perhaps because of a detergent effect on the cell membranes. Interestingly, the Gemini analogue of vector **24** (Fig. 4) is a poor transfectant, revealing a positive macrocyclic effect. Although the results obtained with lower rim guanidinocalix[4]arenes are already quite rewarding, we are confident that improvements in the transfection ability of calixarene-based synthetic vectors can be made by further elaborating the nature of the cationic groups.

Thanks to their delivery properties, our guanidinocalixarenes were also exploited as amphiphilic counterion activators for DNA,[47] in order to transform the nucleic acids into transporters of cations which, otherwise, alone or as a sole DNA counterpart, would not be able to cross lipophilic layers. DNA–countercation complexes formed by the interaction of Calf thymus DNA, and upper (**17–19**) and lower rim guanidinocalixarenes (**24–26**) in the cone conformation, act as cation transporters, carrying safrain O and *p*-xylene-bis-pyridinium

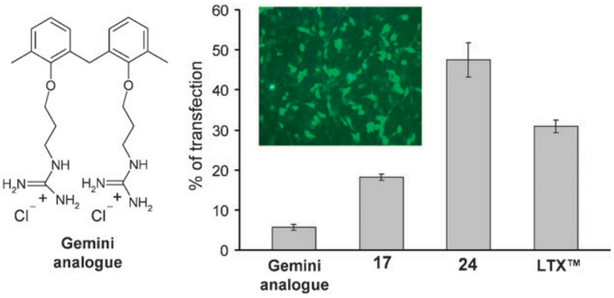
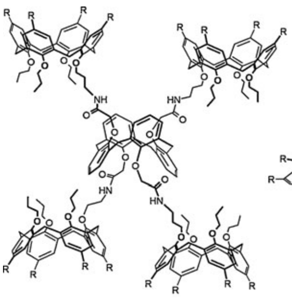


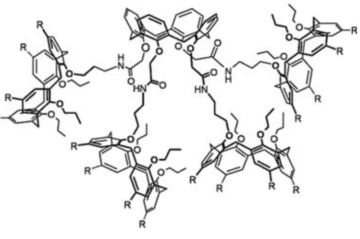
Fig. 4 Transfection efficiency of compound **24** compared to its gemini analogue, the upper rim derivative **17**, and LTX lipofectamine. In the inset, in green, the Rhabdomiosarcoma cells transfected by **24** and detected by fluorescence microscopy upon expression of the Green Fluorescence Protein.

bromide (DPX) across bulk, liquid membranes, and in and out of vesicles through lipid bilayer membranes. Also, in this case, the right balance between hydrophilicity and lipophilicity, which confers a clear amphiphilic character to the ligands, is the key factor for their activity. The conformationally mobile methoxycalix[4]- and -calix[6]arenes **21** and **22** indeed resulted in no activity at all, and the alternate bola-shaped **20** was significantly less active than the cone conformers. The acyclic gemini analogue is more than 100-fold less efficient than calix[4]arene **24**, suggesting that multivalency, macrocyclic preorganization and competing intercalation into DNA are likely to be responsible for the observed calixarene effect. Through a subtle cascade mechanism, the monitoring of the cation transport activity of these DNA–guanidinocalixarene complexes could also be exploited for developing new sensing systems for enzyme activity. In this direction, an on/off switching effect on the transport process due to hydrophilic anions was verified.[47] In particular, using the phytate anion, we observed that it indeed blocks cation transport because of its competitive pairing with the calixarene that cannot interact with and activate the DNA. On the contrary, the process is restored when enzymatic phytate hydrolysis occurs by incubation with phytase, and this also happens in complex matrices.

Multicalixarenes having primary amino groups either directly linked to the aromatic nuclei or as the terminal part of glycine units condensed at the upper rim were prepared by S. E. Matthews and co-workers, and tested as nucleic acid binders and gene vectors.[48] Gel electrophoresis studies indicated that these architectures are able to bind DNA, with the highest efficiency shown by unsymmetrical structure **29**. Nevertheless, effective transfection to CHO cells using pDs2-mito (Clontech) plasmid, leading to expression of a fluorescent protein, was found only with **28** (10% of transfected cells *vs.* 50% observed with commercially available FuGene). This was explained by considering that at physiological pH, probably only the aliphatic amino groups of **28** reach complete protonation, whereas the aromatic NH_2 groups of **27** and **29** do not.



27 R = $NH_3^+Cl^-$
28 R = $NHCOCH_2NH_3^+Cl^-$



29 R = $NH_3^+Cl^-$

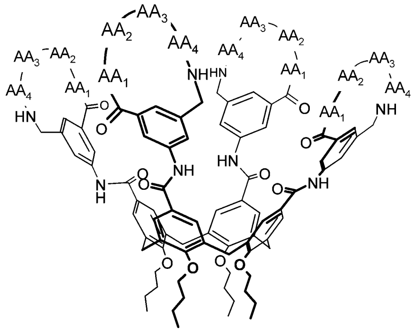
Protein recognition and inhibition

The design of ligands that are able to modulate protein–protein and protein–substrate interactions is an important goal in drug discovery, and also in this field calixarenes are playing a role since the group of A. D. Hamilton used a calix[4]arene platform to synthesize some of them.[49–54] In general, protein recognition and inhibition can be achieved through two different approaches, one aimed at establishing an interaction inside the active binding sites, and the other covering the surface area involved in the biological function.

Inspired by the mode of action and the structural features of antibodies, Hamilton and co-workers chose the second strategy and selected as targets proteins having a distinguished hydrophobic zone surrounded by circular polar regions as an interaction site important for their biological activity. These binding areas can be well matched by the cavity of a calixarene adorned with polar arms terminated with charged and hydrogen bonding residues. Therefore, they attached four pseudo-cyclopentapeptides in a stable hairpin-turn conformation on a cone calix[4]arene with butyl chains at the lower rim. Depending on the amino acid composition of these loops, the ligands showed biological activity towards different proteins. Compound **30**,[49] having the anionic GlyAspGlyAsp sequence, clearly evidenced affinity for the surface of Cytochrome C (Cyt C), which contains a hydrophobic region surrounded by positively-charged Lys side chains around the heme edge, used as an active site and contact area with electron transfer partners. The normal functions of the enzyme, related to the availability of this surface region, is strongly disrupted by the presence of **30** as, for example, the Cyt C redox activity and its interaction with the usual partner Cytochrome C peroxidase.[50] The same antibody mimic also acts as submicromolar inhibitor for α-chymotripsin, which shares some structural features with Cyt C.[51] Following the idea of developing a modular system for the disruption of protein–protein interactions, the same authors replaced an Asp residue of each pseudopeptide loop with a Tyr (**31**), obtaining an antagonist of a platelet-derived growth factor (PDGF) with *in vitro* and *in vivo* antiangiogenic and antitumor activity.[52] Moreover, by substituting in the four loops the anionic Asp units with cationic Lys residues, a selective antagonist (**32**) with nanomolar activity for another growth factor, the vascular endothelial growth factor (VEGF), was produced.[53] This target, in fact, features a hydrophobic region and a patch of anionic residues as a binding surface for its natural receptor. The treatment with peptidocalixarene **32** resulted in the inhibition of angiogenesis, tumorigenesis and metastasis proliferation. A more recent and drastic structural modification of these ligands made by Hamilton and co-workers[54] consists of the replacement of the synthetically demanding pseudopeptide loops with simpler monoacid-monoester isophthalic groups. This change originated a potent pharmacological agent that is able to simultaneously inhibit the activity of PDGF and VEGF, thanks to a preserved complementarity with the protein surfaces. This result puts into question the need for complicated structures in the binding arms, but not the concept of combining multivalency with an apolar cavity, which is indeed maintained in this ligand.

With the same approach, peptidocalix[4]- and -[8]arenes were designed by P. Neri[55] and F. Cunsolo,[56] respectively, for the surface recognition of the enzymes transglutaminase and tryptase.

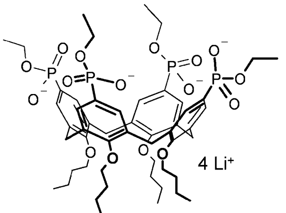
With the aim of targeting enzymes, also G. M. L. Consoli and colleagues synthesized the first example of a calixarene



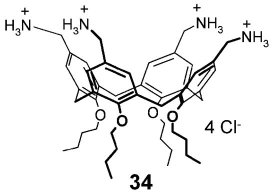
30 AA$_1$ = AA$_3$ = Gly; AA$_2$ = AA$_4$ = Asp
31 AA$_1$ = AA$_3$ = Gly; AA$_2$ = Asp; AA$_4$ = Tyr
32 AA$_1$ = AA$_3$ = Gly; AA$_2$ = AA$_4$ = Lys

functionalized with nucleotides,[57] while V. Kalchenko and co-workers linked phosphonates at the upper rim of a calix[4]arene,[58] thus obtaining inhibitors of a DNA polymerase and a phosphatase, respectively. In both cases, it was reasonably hypothesized that the moieties present in the ligand structure, given their similarity to natural substrates, would compete with them in the binding to the enzyme active site, slowing down its activity. However, data from docking studies suggested that, also in these two cases, contacts between the surface of the protein and the macrocycle would give the main contribution to the interaction. This, in particular, would be the case for the phosphatase, where the active site is actually so deep that it cannot be easily reached by a unit linked to a sterically-demanding calixarene scaffold.

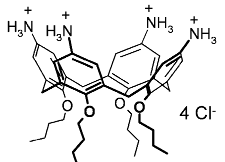
It happens more and more often in macrocyclic and supramolecular chemistry that information derived from studying low molecular weight models is used to target macromolecules. Following this general trend, Schrader and co-workers exploited the recognition properties of tetraphosphonate calix[4]arene **33** towards N/C-protected Arg and Lys in a methanol solution and trapped it in a stearic acid monolayer to obtain a biomimetic protein ligand.[59,60] They found that, while the butyl chains at the lower rim allow inclusion in the monolayer, the anionic head groups protrude from the lipophilic environment into the aqueous solution and are available for the expected charge–charge interactions. When added to the monolayer on water, the calixarene ligands also distribute homogeneously because of the mutual electrostatic repulsion. Nevertheless, they can freely move within the stearic acid layer and self-assemble when polytopic species are present in the aqueous solution to give multiple recognition events. This happens when polyarginines, and even more markedly when proteins characterized by cationic domains, approach the layer surface. Proteins like Histone H1, Cyt C, chymotrypsin, with an isoelectric point pI higher than 7, are detected at 10^{-8}–10^{-9} M concentrations by the phosphonate calixarene **33**. Actually, since only a very small percentage of ligand **33** is embedded in the layer, the recognition mechanism proposed by the authors primarily depends on the calixarenes dissolved in solution, and only subsequently is the whole protein–calixarene complex incorporated into the layer, resulting in the anchoring of the protein and its detection. It is remarkable that even in the presence of an amphiphilic monolayer, where also monovalent ligands such as SDS can in principle constitute multivalent patches, the preorganized multivalency on the calixarene scaffold is superior.



33



34



35

As a complementary extension of these results, tetra-(ammoniummethylene)- and tetraammoniumcalix[4]arenes **34** and **35** were incorporated into the same type of monolayer.[60] With the first ligand, a system able to detect acidic proteins was successfully established. With the second one, due to a partial protonation of the aniline groups at physiological pH, an intermediate behaviour between compounds **33** and **34** was observed, with a recognition ability for both acidic and basic proteins and marked differences in the physical responses depending on the size of the macromolecules. Moreover, the simultaneous presence in the layer of the anionic tetraphosphonate **33** and of one of the two cationic calixarenes allows the obtaining of a system with increased versatility, selectivity and sensitivity at sub-nanomolar levels. The combination of the responses of each protein to the anionic, cationic, mixed and zwitterionic doped monolayers constitutes its corresponding fingerprint. This also generates a new tool for the rapid and valuable detection of proteins from complex mixtures with potential applications in biological, medical and diagnostic fields. This perspective appears even more attractive when these three calixarenes are embedded in lipid/polydiacetylene (PDA) vesicles or films that are able to change colour upon interaction with a protein.[61] "Naked eye" detection was in fact achieved thanks to the different colorimetric responses caused for each tested protein by the specific mutual recognition between the protein surface and the receptor/lipid/PDA vesicle, which is controlled not only by simple electrostatic interactions but also by the overall polarity, size and topology of the protein, and the position of the charged amino acids on its surface.

Further elegant and interesting examples of rational design that exploits rigid cone-shaped calix[4]arenes are those of a clip for destabilized protein assemblies, and of an ion channel stopper, both reported by J. de Mendoza and co-workers.

In the first case,[62] tetra(guanidinomethylene)calixarene **36** was prepared to contrast the effects of mutation in protein p53. The wild-type protein protects cells from tumors, inducing cell arrest when DNA is damaged, triggering the expression of DNA repair machinery or apoptosis when the damage is irreversible. The activity of protein p53 is strongly related to its tetramerization domain (p53TD), controlled by hydrogen bonding, hydrophobic interactions and salt bridges, which determine the formation of a dimer and subsequently a dimer-to-dimer association. Due to a mutation that induces the replacement of each Arg337 by a Hys, the protein (p53-R337H) misses its function of "genome guardian"

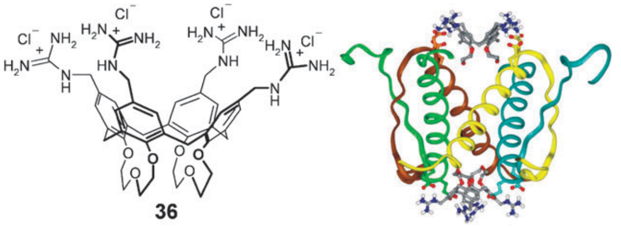
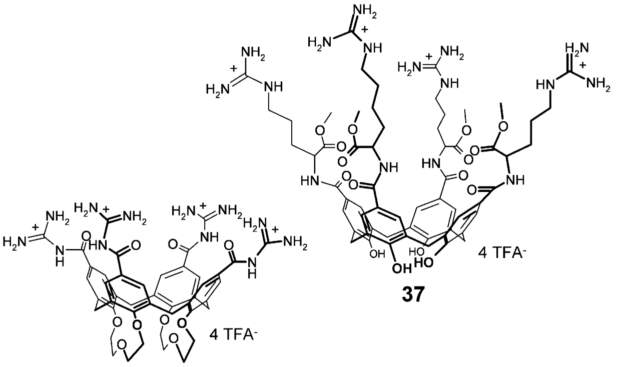

36

Fig. 5 The guanidinocalixarene clip **36** and the model of its complex with p53-R337H (adapted from ref. 62).

and, contextually, the tetrameric assembly is destabilized because of weaker hydrogen bonding network and hydrophobic contacts. Nevertheless, in the tetrameric ensemble of p53-R337H, as in wild type p53, Glu residues belonging to two different monomers form with their carboxylate side chains almost perfect squares above the two ends of the hydrophobic pocket within the strands. These carboxylate groups were selected as suitable counterparts of the guanidinium units of the calixarene. Ligand **36** indeed strongly stabilizes the tetramer assembly of mutant p53-R337H. Thanks to the simultaneous binding of the four guanidinium groups to the Glu carboxylates, and to the presence at the lower rim of the short bridges (which (i) ensure conical shape complementary to the shape of the protein tetramer, (ii) prevent residual conformational freedom of the aromatic rings, improving the preorganization, and (iii) fit into the hydrophobic pocket (Fig. 5)), it can remedy the impaired tetramerization ability of the mutated protein.

Docking studies employing the coordinates of the crystal structure of the human voltage dependent Kv1.2 potassium channel indicated that the conical platform of a calix[4]arene also fits well into the extracellular outer vestibule of this important transmembrane protein.[63] The symmetry of this class of homotetrameric channel seems to match perfectly the macrocycle C_4 symmetry. In fact, also in this second case studied, de Mendoza and co-workers found good channel blockers in compounds **37** and **38**. The positively-charged residues in both of them enhance the complex stability through charge–charge and hydrogen bonding interactions with the Asp side chains present in the extracellular portion of the protein. In particular, compound **37** shows the best complementarity, establishing simultaneously all the possible contacts in the turret loop. This determines a reversible and concentration-dependent inhibition of the channel activity by the calixarene, with suppression of the current flow. Long lipophilic chains at the lower rim of the calixarene were detrimental, since their presence probably confers a detergent feature to the molecule, jeopardizing the integrity of the cells.



37

38

Glycoclusters in lectin recognition and inhibition

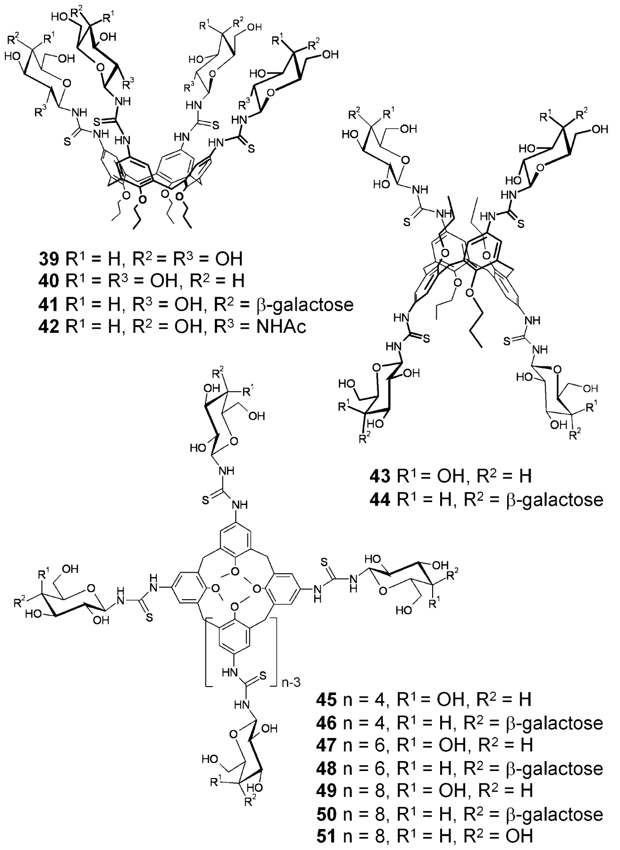
Proteins that bind carbohydrates but do not have enzymatic or immune activity are called lectins.[64,65] In mammalians, they play a fundamental role in numerous processes of a physiological and pathological nature, such as fertilization, cell growth and trafficking, virus and bacteria and toxin adhesion to cell membranes, tumour and metastasis growth .[66] There is a specific language, called the *Sugar Code*, through which communication between lectins and carbohydrates is ensured.[67] The action of lectins towards polysaccharides, glycoproteins and glycolipids is very often characterized by the onset of the multivalent effect, which is so important for carbohydrates that it assumes the specific name of the *glycoside cluster effect*.[68] In the last few years, many synthetic polyglycosylated systems have been designed to interfere with these processes as competitors of natural substrates, with the aim of obtaining a deeper insight into these phenomena and new biologically active compounds.[69] Due to their oligomeric nature, calixarenes were also introduced in the world of glycoscience. Compared to other scaffolds, the general structural and conformational features of calixarenes mentioned above allow the assembly of small libraries where ligand size, geometry, valency and the display of the binding units can be subtly modulated. This makes the investigation of the structure–activity relationship feasible and helps in the understanding of the factors that influence the recognition processes of the carbohydrate binding proteins at a molecular level. Moreover, these options provide the possibility to build up clusters optimized for each family and type of lectin. Some of these proteins are in fact non-covalent assemblies with the number of monomers possibly changing with the conditions; others have several carbohydrate binding domains that are covalently linked. Topologically, some have the binding sites positioned on the same face, while others project them in opposite directions. This may lead to the simultaneous interaction between several lectin units and the multivalent ligands, causing extensive crosslinking and the consequent formation of complex networks that might evolve in agglutination.

The first examples of calixarenes functionalized with carbohydrates at the upper and lower rim were reported by us.[70] In these neoglycoconjugates, glucose, galactose and lactose units were linked to a calix[4]arene structure through classical glycosylation reactions.[71] At that time, we were primarily interested in introducing these natural moieties onto the calixarene scaffold, the obvious necessary step for the development of glycoclusters based on these macrocycles. Since then, we[72] and others[73,74] have successfully applied other glycoconjugation reactions, widening the scope of glycoclusters based on calixarenes. It is notable that the nature and length of the linker between the macrocycle and the carbohydrate unit can influence the binding properties, and also the natural selectivity of the lectin (for example, between the two anomers of the sugar substrate) in a way that is not easy to foresee.

Soon after our first reports, R. Roy, sharing the same positive view on the potential of calixarenes in this field, prepared some glycoclusters of different valency linking sialic acid[75] and *N*-acetyl-galactosamine[76] units to the lower rim of *p-tert*-butylcalix[4]arene in the cone conformation. In addition to the synthesis, he tested the activity of these multivalent systems towards lectins specific to the saccharides present in the structures. By turbidimetric assays, the sialylated compound showed a strong crosslinking activity towards the tetrameric wheat germ agglutinin (WGA). The same behaviour was observed for compounds adorned with GalNAc towards *Vicia Villosa* Agglutinin, which is also inhibited in its binding to asialoglycophorin, a natural glycoprotein of human erytrocytes. However, in this latter study, a real cluster effect was not observed, since the increase of the activity with respect to a monomeric model was lower than that expected on the basis of the number of glycoside units present in the ligand. In all these cases, the cross-linking ability of the polyglycosylated calixarenes is inhibited by the corresponding monosaccharide, demonstrating the specificity of the binding to the lectin.

In order to facilitate the synthesis of these systems, we successfully tried the coupling reaction between aminocalixarenes and glycosylisothiocyanates. Through this reaction, we[77,78] and others[79] have efficiently prepared, over several years, a variety of multivalent calixarene glycoclusters (Fig. 6) having different shapes and conformational mobilities.

The thiourea-linked calixarene neoglycoconjugates show specific binding properties towards vegetal lectins such as Concanavalin A (ConA) for the *gluco* derivatives[77,78] (*e.g.* **39**), Peanut Lectin for the *galacto* derivatives (*e.g.* **40**),[77] and WGA for the *N*-acetyl-glucosamine (GlcNAc) clusters[80] (*e.g.* **42**), inhibiting their hemagglutination ability. The strength of the interaction was qualitatively indicated by the need for a very large excess of the monomers to disrupt, sometimes only partially, the aggregates and by the formation of precipitates upon addition of the glycoclusters to the lectin solutions. We also detected the agglutination of ConA due to our thioureidocalixarenes by AFM[78] on mica. After increasing incubation times, solution mixtures of the ligand and protein showed the presence of progressively larger aggregates. In some experiments, the positive effect of the multiple exposition of ligand units was clearly revealed, even if too-crowded clusters gave negative results, probably because the sugar units could not be exposed properly.[80] Moreover, a quite relevant aspect to consider is the tendency of these compounds to self-aggregate in water. This behaviour was observed not only for amphiphilic cone glycocalix[4]arenes, where a well-defined separation occurs between the polar heads and the apolar cavity plus alkyl chains, but also for mobile calix[6]- and -[8]arenes, even with simple methyl groups at the lower rim. The formation of aggregates was evident by simply observing the broadening of the NMR signals of these compounds in D_2O. More accurate studies, using dynamic light scattering and AFM confirmed that they give rise to micelles, vesicles or to a mixture of the two forms in equilibrium,[81] depending on the conditions. Also, the dimensions of these aggregates can dramatically change, for instance, in absence or presence of phosphate buffer, as we were able to observe with octamethoxy-octaglucosylthioureidocalix[8]arene **51**[78] (Fig. 7). Obviously, the aggregation can affect the recognition process with lectins, in particular with regard to the definition of the actual cluster valency at work.



39 R^1 = H, R^2 = R^3 = OH
40 R^1 = R^3 = OH, R^2 = H
41 R^1 = H, R^3 = OH, R^2 = β-galactose
42 R^1 = H, R^2 = OH, R^3 = NHAc

43 R^1 = OH, R^2 = H
44 R^1 = H, R^2 = β-galactose

45 n = 4, R^1 = OH, R^2 = H
46 n = 4, R^1 = H, R^2 = β-galactose
47 n = 6, R^1 = OH, R^2 = H
48 n = 6, R^1 = H, R^2 = β-galactose
49 n = 8, R^1 = OH, R^2 = H
50 n = 8, R^1 = H, R^2 = β-galactose
51 n = 8, R^1 = H, R^2 = OH

Fig. 6 Examples of glycosylthioureidocalix[n]arenes.

In perspective, another interesting property of these glycocalixarenes is related to the thiourea units, which can act as hydrogen bonding donors and cooperate with the macrocyclic cavity for the complexation of small substrates, ideally molecules with pharmacological activity. Solution experiments by NMR[77] and gas phase studies by ESI mass spectrometry[82] effectively gave evidence of the binding of small organic molecules such as amino acids, nucleotides and phosphate containing compounds, with some preference for those having aromatic tails. Although more data must be collected, the possibility of developing "intelligent" drug delivery systems, selective for a specific organ/tissue thanks to the glycoside moieties,[83] is an attractive feature of calixarene glycoclusters.

Moving towards examples of medically-relevant carbohydrate binding proteins, in collaboration with H.-J. Gabius and S. André, we have prepared and studied a small library of galacto- and lactoclusters as inhibitors of human galectins.[84] Gal-1, Gal-3 and Gal-4 were selected as representatives of the different topologies of this family of lectins that are selective for galactose residues.[85] The small library of tested compounds was based on calixarene derivatives having different conformations, sizes, mobilities, geometries and valencies. Lactose-containing ligands are more efficient both in solid phase assays, by inhibiting the binding of galectins to anchored asialofetuin, and in bioassays with cells, inhibiting the binding of the same lectins to suitably-selected human tumour cell lines. The results we obtained, mainly in this second type of

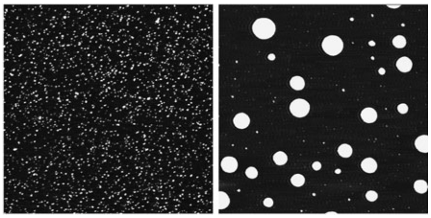


Fig. 7 AFM images in tapping mode on air (2×2 μm scans) of octamethoxy-octaglucosylthioureidocalix[8]arene **51** 5×10^{-4} M (left) in water solution and (right) in phosphate buffer (0.1 M, pH 7).

test, are remarkable for two reasons. On the one hand, we observed a conspicuous degree of inhibition, in particular with hexa- and octalactose derivatives **48** and **50** against the binding of tandem repeat-type Gal-4 to human pancreatic carcinoma cells (Fig. 8). Compared to lactose, which does not show any inhibition activity, even at 10 mM, the two clusters have IC_{50} values at submicromolar levels considering the sugar concentration, which not only represents the strongest activity reported so far towards this galectin, but also indicates a marked multivalent effect. On the other hand, we found a prominent selectivity among our calixarenes in the inhibition of the different classes of galectins. This clearly indicates that no unspecific binding is occurring and that the structural features of the clusters play a fundamental role in the interaction with the structurally different galectins. The most significant example is the behaviour of cone tetralactosylthioureidocalix[4]arene **41** and its analogue **44** in the 1,3-alternate conformation. These compounds are the best and the worst ligands, respectively, for Gal-3, whereas the opposite is true for Gal-1. Moreover, the good affinity shown by cluster **44** both for Gal-1 and Gal-4 (Fig. 8) places this glycocalixarene in the condition of being an interesting simultaneous inhibitor for these two proteins, which are involved together in some pathological events.

Some GlcNAc substituted thioureidocalix[4]arenes were shown by V. Kren and co-workers to stimulate the natural killer (NK) cell-mediated antitumor immune response.[86] NKR-P1 and CD69, two lectin-like receptors expressed on the surface of NK cells in rats and humans, respectively, are the targets of these glycoclusters, which, in the case of cone derivative **39**, exhibit IC_{50} values at a nanomolar level.

As mentioned above, many microorganisms and a number of their excreted products adhere to the cell membrane of the host through multivalent interactions with the saccharide portion of surface glycoproteins or glycolipids. Classical is the case of the cholera toxin (CT), a toroid-like AB_5 protein whose B sub-units expose five identical recognition sites on the same face for the binding to the ganglioside GM1 pentasaccharide (o-GM1). In collaboration with A. Bernardi, we designed[87] a potent divalent inhibitor (**52**) of this toxin by the combination of a calix[4]arene scaffold and a mimic of the GM1 saccharide (pseudo-GM1), where lactose and sialic acid are replaced by cyclohexandiol and *R*-lactic acid, respectively (Fig. 9). Differently from other glycocalixarenes here-reported, the spacer between the cavity and the ligand units is significantly longer to allow the simultaneous interaction of the epitopes, even with two non-adjacent binding sites belonging to the same pentameric protein. In fact, the geometry and the binding site orientation of CT allow the formation of a 1 : 1 lectin : cluster complex. Although the monomeric pseudo-GM1 interacts with the toxin with a dissociation constant K_d = 190 μM *vs.* a K_d = 219 nM of o-GM1, our divalent system (**52**) was found to be very efficient by means of fluorimetric titrations and Elisa tests. A K_d = 48 nM at 50% toxin saturation was determined, and this value is noteworthy, not only because it is lower than that of natural o-GM1, but mainly for the remarkable affinity enhancement factor of close to 4000 (2000 fold per sugar mimic) with respect to the single pseudo-GM1. These data suggest that, despite the distance of the two sugar mimic units from the calixarene scaffold, the multivalent recognition process also occurs efficiently thanks to the contribution of the linkers, as evidenced by STD and NOE-difference NMR experiments.

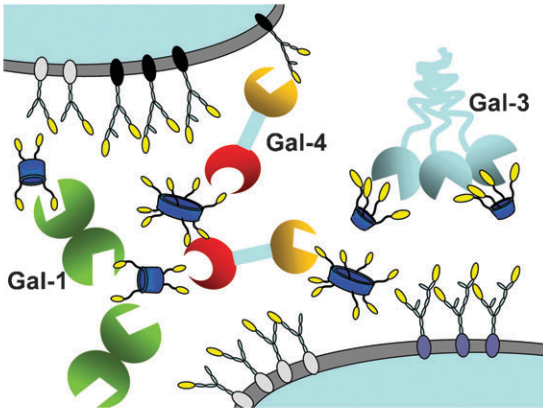


Fig. 8 Schematic representation of the cell binding inhibition by glycocalixarenes towards three different types of galectins.

Through the application of a typical "click chemistry" reaction, the Cu(I)-catalyzed 1,3-dipolar cycloaddition between alkyne and azide groups, to the conjugation of saccharides to calixarene scaffolds, A. Dondoni and A. Marra have produced in the last few years a wide number of multivalent glycosylated macrocycles.[88–91] Recently, they tested some of their ligands against viruses[89] and bacteria.[90] The activity of polysialylated calix[4]arenes in the cone conformation with different valencies was first evaluated against the BK virus by hemagglutination inhibition, but they did not show any particular efficiency. Nevertheless, the pre-incubation of the virus with these synthetic clusters before its administration to Vero cells neutralizes the infectivity and blocks the cytopatic effect caused by the pathogen. Comparable results were observed against the influenza A virus in experiments characterized by a moderate glycoside cluster effect.

To fight PA-IL bacterial lectin from *Pseudomonas aeruginosa*, responsible for lung infections in cystic fibrosis patients, Matthews and co-workers recently tested lower rim galactosylated calix[4]arenes in different conformational arrangements.[92] The most important contribution of this work is the ITC analysis of the binding processes between this calcium dependent tetrameric protein and a series of glycocalix[4]arenes, in which valency and ligand orientation were varied. A favourable balance between enthalpic and entropic contributions, also due to the glycoside cluster effect, which increases by increasing

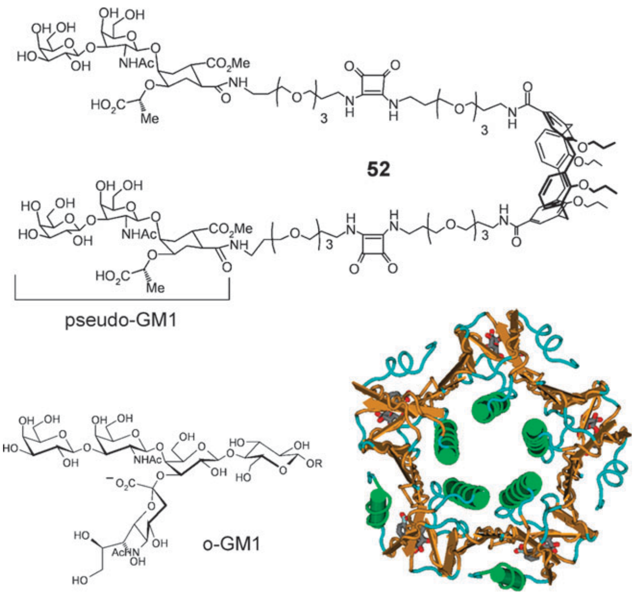


Fig. 9 Divalent calix[4]arene based inhibitor **52** of Cholera Toxin adorned with two units of o-GM1 mimic (pseudo GM1), o-GM1 and CT-B$_5$ pentamer structure.

the number of galactose units, determines an efficient binding. Indeed, all glycoclusters were more efficient than the monomeric reference compound, the best one being the tetravalent derivative in the 1,3-alternate conformation with a K_d at a nanomolar level. Despite the potential for extensive cross-linking, only traces of precipitate were observed during the ITC measurements, suggesting that discrete aggregates are mainly formed.

Another important direction for the development of glycoclusters is that of carbohydrate-based vaccines, although this topic must be tackled with care because of the possibility of autoimmune responses towards saccharides, which are often self-antigens for humans. In this context, C. Geraci and co-workers have produced the only attempt based on a calixarene scaffold known up to now, by preparing a compound where four GalNAc-Cys units are linked at the upper rim of a cone calix[4]arene to mimic the clustered motif of the tumour associated Tn antigen.[93] Moreover, a lipopeptide as immunoadjuvant was introduced at the lower rim of this derivative to support the usually low immunogenicity of simple carbohydrates. Mice administered with this derivative gave rise to a significant production of antibodies, substantially larger than that caused by a monovalent reference compound. Once again, the clustered construct demonstrated a biological activity higher than the sum of those due to the single moieties, apparently mimicking more properly the natural substrates and providing, in this specific case, a potential vaccine candidate.

Binding to other bio-macromolecules

Amphiphilic calix[4]arenes with alkyl chains at the upper rim and positively-charged groups at the lower rim (Fig. 10) were successfully proposed by K. H. Mayo as topomimetics of amphipathic β-sheet and helix-forming bactericidal peptides for the neutralization of lipopolysaccharide (LPS) endotoxins.[94,95] The LPS structure is typically characterized by the presence of the conserved phospholipid moiety, called lipid A, and of a highly variable polysaccharide chain. Well-known peptides able to block LPS activity by interacting with the phospholipidic part form helices or sheets with well-defined polar and apolar faces that are covalently assembled into an amphipathic structure. These features were easily reproduced in non-peptidic ligands **53** based on the calixarene scaffold, mimicking a small portion of the helix or β-sheet. *In vitro*, they show strong activity against different LPS species, higher than that of the peptides used as references. IC50 values in the nanomolar range were found and, when tested *in vivo* in mice administered with a lethal dose of LPS, they demonstrated a good protection efficacy, determining a 25–100% survival, depending on the microbial origin of LPS.

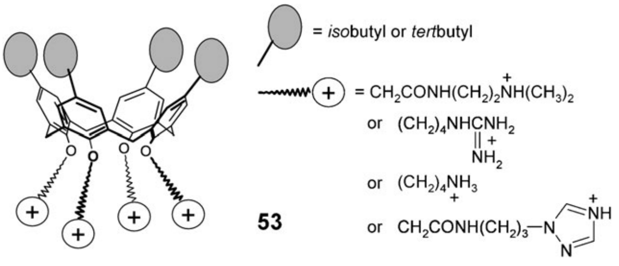


Fig. 10 Amphiphilic calix[4]arenes for LPS inhibition.

Although a definitive proof of the mode of binding of these compounds to lipid A was not reported, the authors reasonably proposed that the cationic head groups at the lower rim interact with the negatively-charged phosphates, while the alkyl chains at the upper rim are involved in hydrophobic binding with the lipophilic alkyl tails. With respect to the activity shown, some general structural requirements can be identified on the basis of the NMR and biological data collected for all the members of the library. In particular, the presence of (i) lipophilic chains at the upper rather than the lower rim, (ii) four positively-charged groups rather than two and (iii) guanidinium or primary amines rather than tertiary amines or triazole groups were found to be important. Thanks to the good results obtained, the authors proposed these derivatives as potential new weapons to fight endotoxemia and septic shock caused by LPS.

Finally, positively charged aminocalix[8]arenes mentioned above as tryptase inhibitors also deserve a citation in this last section because they represent the sole example, to the best of our knowledge, of calixarenes studied for the binding to polysaccharides.[96] Cunsolo and co-workers, in fact, tested these molecules as heparin binders and obtained very interesting results. Indeed, these polycationic ligands show much better binding compared to protamine and polylysine widely used as heparin antagonists. These results were attributed to the peculiar conformational properties of the calix[8]arene scaffold, which would ensure, with respect to the low ordered polylysine and the conformationally rigid protamine, a better mutual adaptability between the ligand and the polysaccharide chain, causing efficient charge neutralization both in aqueous solution and in blood.

Conclusions

Through the selected examples reported in this review, we have highlighted the important role played by calixarenes in tackling problems of biological interest and, for each chapter, we have indicated some perspectives. It appears clear that the synthetic versatility of these macrocycles, the possibility of controlling their conformational properties and three-dimensional shape, their multivalent nature when considered as scaffolds (multi)functionalized at the lower and upper rim, and even (although less commonly) at the methylene bridges and, not least, their lack of toxicity, are all attractive features for using calixarenes in biological and biomedical fields. Through a retrospective ''bird's eye'' view of this research topic over the last ten years, a trend can clearly be evidenced for this class of macrocycles, moving from biomimetic receptors to multivalent ligands for biomacromolecules. In the first case, relatively simple calixarene hosts, adorned with hydrogen bonding donor or acceptor units, are used as mimics of more complex biological functions such as recognition, transport and catalysis. Here, the apolar cavity and the conformational preferences of the calixarenes (mainly calix[4]-) are exploited to modulate efficiency and selectivity, often obtaining quite impressive results. In the second case, the calixarene ligands elicit a cellular response or directly interfere with it, as shown, for example, for α-chymotripsin, vascular endothelial growth factor (VEGF) and Kv1.2 potassium channel inhibition, intergalectin selection, cell transfection and pathogen fighting. The examples illustrated in this review refer only to a few of the many biological processes that can be targeted by synthetic weapons for therapeutic or mechanistic purposes. Therefore, it is easy to forecast that calixarenes will have a bright future in this context.

Acknowledgements

The authors are grateful to Fondazione Cassa di Risparmio di Parma and the Ministero dell'Istruzione, dell'Università e della Ricerca (MIUR, PRIN 2008 projects no. 2008HZJW2L and 200858SA98) for financial support.

References

1 C. D. Gutsche, *Calixarenes: An Introduction*, RSC Publishing, Cambridge, UK, 2nd edn, 2008.
2 V. Böhmer, *Angew. Chem., Int. Ed. Engl.*, 1995, **34**, 713.
3 *Calixarenes in Action*, ed. L. Mandolini and R. Ungaro, Imperial College Press, London, 2000.
4 *Calixarenes 2001*, ed. Z. Asfari, V. Böhmer, J. Harrowfield and J. Vicens, Kluwer Academic Publishers, Dordrecht, 2001.
5 *Calixarenes in the Nanoworld*, ed. J. Vicens and J. Harrowfield, Springer, Dordrecht, 2007.
6 A. Mulder, T. Auletta, A. Sartori, S. Del Ciotto, A. Casnati, R. Ungaro, J. Huskens and D. N. Reinhoudt, *J. Am. Chem. Soc.*, 2004, **126**, 6627.
7 A. Arduini, D. Demuru, A. Pochini and A. Secchi, *Chem. Commun.*, 2005, 645.
8 A. Arduini, F. Ciesa, M. Fragassi, A. Pochini and A. Secchi, *Angew. Chem., Int. Ed.*, 2005, **44**, 278.
9 D. M. Rudkevich, *Eur. J. Org. Chem.*, 2007, 3255.
10 D. M. Rudkevich, *Bull. Chem. Soc. Jpn.*, 2002, **75**, 393.
11 R. V. Rodik, V. I. Boyko and V. I. Kalchenko, *Curr. Med. Chem.*, 2009, **16**, 1630.
12 F. Perret, A. N. Lazar and A. W. Coleman, *Chem. Commun.*, 2006, 2425.
13 J.-M. Lehn, *Supramolecular Chemistry: Concepts and Perspectives*, VCH, Weinheim, 1985.
14 A. Casnati, M. Fabbi, N. Pelizzi, A. Pochini, F. Sansone, R. Ungaro, E. Di Modugno and G. Tarzia, *Bioorg. Med. Chem. Lett.*, 1996, **6**, 2699.
15 A. P. Davis, *Org. Biomol. Chem.*, 2009, **7**, 3629.
16 S. Kubik, *Angew. Chem., Int. Ed.*, 2009, **48**, 1722.
17 M. Mazik, *ChemBioChem*, 2008, **9**, 1015.
18 G. Das and A. D. Hamilton, *Tetrahedron Lett.*, 1997, **38**, 3675.
19 A. S. Droz, U. Neidlein, S. Anderson, P. Seiler and F. Diederich, *Helv. Chim. Acta*, 2001, **84**, 2243.
20 M. Segura, B. Bricoli, A. Casnati, E. M. Munoz, F. Sansone, R. Ungaro and C. Vicent, *J. Org. Chem.*, 2003, **68**, 6296.
21 T. M. Fyles, *Chem. Soc. Rev.*, 2007, **36**, 335.
22 A. P. Davis, D. N. Sheppard and B. D. Smith, *Chem. Soc. Rev.*, 2007, **36**, 348.
23 S. Licen, F. De Riccardis, I. Izzo and P. Tecilla, *Curr. Drug Discovery Technol.*, 2008, **5**, 86.
24 G. W. Gokel and N. Barkey, *New J. Chem.*, 2009, **33**, 947.
25 S. J. Iqbal Khayzuran and P. J. Cragg, *Dalton Trans.*, 2007, 26.
26 B. S. Creaven, D. F. Donlon and J. McGinley, *Coord. Chem. Rev.*, 2009, **253**, 893.
27 T. J. Jentsch, T. Maritzen and A. A. Zdebik, *J. Clin. Invest.*, 2005, **115**, 2039.
28 V. Sidorov, F. W. Kotch, G. Abdrakhmanova, R. Mizani, J. C. Fettinger and J. T. Davis, *J. Am. Chem. Soc.*, 2002, **124**, 2267.
29 J. L. Seganish, P. V. Santacroce, K. J. Salimian, J. C. Fettinger, P. Zavalij and J. T. Davis, *Angew. Chem., Int. Ed.*, 2006, **45**, 3334.
30 I. Izzo, S. Licen, N. Maulucci, G. Autore, S. Marzocco, P. Tecilla and F. De Riccardis, *Chem. Commun.*, 2008, 2986.
31 N. Maulucci, F. De Riccardis, C. B. Botta, A. Casapullo, E. Cressina, M. Fregonese, P. Tecilla and I. Izzo, *Chem. Commun.*, 2005, 1354.
32 K. S. J. Iqbal, M. C. Allen, F. Fucassi and P. J. Cragg, *Chem. Commun.*, 2007, 3951.
33 A. J. Kirby and F. Hollfelder, *From Enzyme Models to Model Enzymes*, RSC, Cambridge, 2009.
34 P. Molenveld, J. F. J. Engbersen and D. N. Reinhoudt, *Chem. Soc. Rev.*, 2000, **29**, 75.
35 R. Cacciapaglia, A. Casnati, L. Mandolini, D. N. Reinhoudt, R. Salvio, A. Sartori and R. Ungaro, *J. Org. Chem.*, 2005, **70**, 624.
36 R. Cacciapaglia, A. Casnati, L. Mandolini, D. N. Reinhoudt, R. Salvio, A. Sartori and R. Ungaro, *J. Am. Chem. Soc.*, 2006, **128**, 12322.
37 R. Cacciapaglia, A. Casnati, L. Mandolini, A. Peracchi, D. N. Reinhoudt, R. Salvio, A. Sartori and R. Ungaro, *J. Am. Chem. Soc.*, 2007, **129**, 12512.
38 M. Mammen, S.-K. Choi and G. M. Whitesides, *Angew. Chem., Int. Ed.*, 1998, **37**, 2755.
39 P. I. Kitov and D. R. Bundle, *J. Am. Chem. Soc.*, 2003, **125**, 16271.
40 G. Ercolani, *J. Am. Chem. Soc.*, 2003, **125**, 16097.
41 Y. H. Shi and H.-J. Schneider, *J. Chem. Soc., Perkin Trans. 2*, 1999, 1797.
42 R. Zadmard and T. Schrader, *Angew. Chem., Int. Ed.*, 2006, **45**, 2703.
43 P. Shahgaldian, M. A. Sclotti and U. Pieles, *Langmuir*, 2008, **24**, 8522.
44 M. Dudic, A. Colombo, F. Sansone, A. Casnati, G. Donofrio and R. Ungaro, *Tetrahedron*, 2004, **60**, 11613.
45 F. Sansone, M. Dudic, G. Donofrio, C. Rivetti, L. Baldini, A. Casnati, S. Cellai and R. Ungaro, *J. Am. Chem. Soc.*, 2006, **128**, 14528.
46 V. Bagnacani, F. Sansone, G. Donofrio, L. Baldini, A. Casnati and R. Ungaro, *Org. Lett.*, 2008, **10**, 3953.
47 T. Takeuchi, V. Bagnacani, F. Sansone and S. Matile, *ChemBioChem*, 2009, **10**, 2793.
48 R. Lalor, J. L. Di Gesso, A. Mueller and S. E. Matthews, *Chem. Commun.*, 2007, 4907.
49 Y. Hamuro, M. Crego-Calama, H. S. Park and A. D. Hamilton, *Angew. Chem., Int. Ed. Engl.*, 1997, **36**, 2680.
50 Y. Wei, G. L. McLendon, A. D. Hamilton, M. A. Case, C. B. Purring, Q. Lin, H. S. Park, C. S. Lee and T. N. Yu, *Chem. Commun.*, 2001, 1580.

51 H. S. Park, Q. Lin and A. D. Hamilton, *J. Am. Chem. Soc.*, 1999, **121**, 8.

52 S. M. Sebti and A. D. Hamilton, *Oncogene*, 2000, **19**, 6566.

53 J. Z. Sun, M. A. Blaskovich, R. K. Jain, F. Delarue, D. Paris, S. Brem, O. M. Wotoczek, Q. Lin, D. Coppola, K. H. Choi, M. Mullan, A. D. Hamilton and S. M. Sebti, *Cancer Res.*, 2004, **64**, 3586.

54 J. Z. Sun, D. A. Wang, R. K. Jain, A. Carie, S. Paquette, E. Ennis, M. A. Blaskovich, L. Baldini, D. Coppola, A. D. Hamilton and S. M. Sebti, *Oncogene*, 2005, **24**, 4701.

55 S. Francese, A. Cozzolino, L. Caputo, C. Esposito, M. Martino, C. Gaeta, F. Troisi and P. Neri, *Tetrahedron Lett.*, 2005, **46**, 1611.

56 T. Mecca, G. M. L. Consoli, C. Geraci and F. Cunsolo, *Bioorg. Med. Chem.*, 2004, **12**, 5057.

57 G. M. L. Consoli, G. Granata, E. Galante, I. Di Silvestro, L. Salafia and C. Geraci, *Tetrahedron*, 2007, **63**, 10758.

58 A. I. Vovk, L. A. Kononets, V. Y. Tanchuk, S. O. Cherenok, A. B. Drapailo, V. I. Kalchenko and V. P. Kukhar, *Bioorg. Med. Chem. Lett.*, 2010, **20**, 483.

59 R. Zadmard, M. Arendt and T. Schrader, *J. Am. Chem. Soc.*, 2004, **126**, 7752.

60 R. Zadmard and T. Schrader, *J. Am. Chem. Soc.*, 2005, **127**, 904.

61 S. Kolusheva, R. Zadmard, T. Schrader and R. Jelinek, *J. Am. Chem. Soc.*, 2006, **128**, 13592.

62 S. Gordo, V. Martos, E. Santos, M. Menendez, C. Bo, E. Giralt and J. de Mendoza, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 16426.

63 V. Martos, S. C. Bell, E. Santos, E. Y. Isacoff, D. Trauner and J. de Mendoza, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 10482.

64 H. Lis and N. Sharon, *Chem. Rev.*, 1998, **98**, 637.

65 H. J. Gabius, S. André, H. Kaltner and H. C. Siebert, *Biochim. Biophys. Acta, Gen. Subj.*, 2002, **1572**, 165.

66 H. J. Gabius, H. C. Siebert, S. André, J. Jimenez-Barbero and H. Rudiger, *ChemBioChem*, 2004, **5**, 740.

67 *The Sugar Code*, ed. H.-J. Gabius, Wiley-Blackwell, Weinheim, 2009.

68 Y. C. Lee and R. T. Lee, *Acc. Chem. Res.*, 1995, **28**, 321.

69 H. Rudiger, H. C. Siebert, D. Solis, J. Jimenez-Barbero, A. Romero, C. W. der Lieth, T. Diaz-Maurino and H. J. Gabius, *Curr. Med. Chem.*, 2000, **7**, 389.

70 A. Marra, M. C. Scherrmann, A. Dondoni, A. Casnati, P. Minari and R. Ungaro, *Angew. Chem., Int. Ed. Engl.*, 1995, **33**, 2479.

71 A. Dondoni, A. Marra, M. C. Scherrmann, A. Casnati, F. Sansone and R. Ungaro, *Chem.–Eur. J.*, 1997, **3**, 1774.

72 U. Schädel, F. Sansone, A. Casnati and R. Ungaro, *Tetrahedron*, 2005, **61**, 1149.

73 A. Marra, A. Dondoni and F. Sansone, *J. Org. Chem.*, 1996, **61**, 5155.

74 A. Dondoni, M. Kleban, X. Hu, A. Marra and H. D. Banks, *J. Org. Chem.*, 2002, **67**, 4722.

75 S. J. Meunier and R. Roy, *Tetrahedron Lett.*, 1996, **37**, 5469.

76 R. Roy and J. M. Kim, *Angew. Chem., Int. Ed.*, 1999, **38**, 369.

77 F. Sansone, E. Chierici, A. Casnati and R. Ungaro, *Org. Biomol. Chem.*, 2003, **1**, 1802.

78 F. Sansone, L. Baldini, A. Casnati and R. Ungaro, *Supramol. Chem.*, 2008, **20**, 161.

79 G. M. L. Consoli, F. Cunsolo, C. Geraci, T. Mecca and P. Neri, *Tetrahedron Lett.*, 2003, **44**, 7467.

80 G. M. L. Consoli, F. Cunsolo, C. Geraci and V. Sgarlata, *Org. Lett.*, 2004, **6**, 4163.

81 N. Micali, V. Villari, G. M. L. Consoli, F. Cunsolo and C. Geraci, *Phys. Rev. E: Stat., Nonlinear, Soft Matter Phys.*, 2006, **73**, 051904-1.

82 M. Torvinen, R. Neitola, F. Sansone, L. Baldini, R. Ungaro, A. Casnati, P. Vainiotalo and E. Kalenius, *Org. Biomol. Chem.*, 2010, **8**, 906.

83 H. J. Gabius, *Adv. Drug Delivery Rev.*, 2004, **56**, 421.

84 S. André, F. Sansone, H. Kaltner, A. Casnati, J. Kopitz, H. J. Gabius and R. Ungaro, *ChemBioChem*, 2008, **9**, 1649.

85 H. J. Gabius, *Eur. J. Biochem.*, 1997, **243**, 543.

86 K. Krenek, M. Kuldova, K. Hulikova, I. Stibor, P. Lhotak, M. Dudic, J. Budka, H. Pelantova, K. Bezouska, A. Fiserova and V. Kren, *Carbohydr. Res.*, 2007, **342**, 1781.

87 D. Arosio, M. Fontanella, L. Baldini, L. Mauri, A. Bernardi, A. Casnati, F. Sansone and R. Ungaro, *J. Am. Chem. Soc.*, 2005, **127**, 3660.

88 A. Dondoni and A. Marra, *J. Org. Chem.*, 2006, **71**, 7546.

89 A. Marra, L. Moni, D. Pazzi, A. Corallini, D. Bridi and A. Dondoni, *Org. Biomol. Chem.*, 2008, **6**, 1396.

90 L. Moni, G. Pourceau, J. Zhang, A. Meyer, S. Vidal, E. Souteyrand, A. Dondoni, F. Morvan, Y. Chevolot, J. J. Vasseur and A. Marra, *ChemBioChem*, 2009, **10**, 1369.

91 A. Vecchi, B. Melai, A. Marra, C. Chiappe and A. Dondoni, *J. Org. Chem.*, 2008, **73**, 6437.

92 S. Cecioni, R. Lalor, B. Blanchard, J. P. Praly, A. Imberty, S. E. Matthews and S. Vidal, *Chem.–Eur. J.*, 2009, **15**, 13232.

93 C. Geraci, G. M. L. Consoli, E. Galante, E. Bousquet, M. Pappalardo and A. Spadaro, *Bioconjugate Chem.*, 2008, **19**, 751.

94 X. M. Chen, R. P. M. Dings, I. Nesmelova, S. Debbert, J. R. Haseman, J. Maxwell, T. R. Hoye and K. H. Mayo, *J. Med. Chem.*, 2006, **49**, 7754.

95 R. R. M. Dings, X. M. Chen, D. M. E. I. Hellebrekers, L. I. van Eijk, Y. Zhang, T. R. Hoye, A. W. Griffioen and K. H. Mayo, *J. Natl. Cancer Inst.*, 2006, **98**, 932.

96 T. Mecca, G. M. L. Consoli, C. Geraci, R. La Spina and F. Cunsolo, *Org. Biomol. Chem.*, 2006, **4**, 3763.