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Exploring Chemical Space with Aptamers

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For many years, my research group has developed and applied aptamer- and allosteric ribozyme-based technologies for various purposes in diagnostics, biosensing, chemistry, and molecular biomedicine.[1] Aptamers are single-stranded synthetic oligonucleotides that can adopt well-defined three-dimensional shapes.[2] These consist of binding pockets and clefts that enable this class of functional nucleic acids to specifically recognize and tightly bind to a huge variety of different molecular targets,[3] ranging from simple ones such as small organic molecues,[4] ions, proteins, nucleic acids,[5] and other macromolecules to complex targets like higher order protein complexes, whole cells, viruses, parasites, or tissues. Consequently, aptamers possess properties comparable to those of proteins or antibodies and thus provide a useful surrogate to conventional protein-based biochemical or biomedicinal tools for research and diagnostics (Figure 1).

There are several ways of how aptamers can be isolated and that have been further developed based on the classical in vitro selection or SELEX[a]-process; recent examples include selections by kinetic capillary electrophoresis or approaches called non-SELEX, post-SELEX, tailored-SELEX, genomic SELEX, or FACS-SELEX.[6] Common to all aptamer-selection techniques is the isolation of functional nucleic acids from large libraries covering a huge sequence space of up to $10^{15}-10^{16}$ different sequences, a diversity that has not been accomplished by any other type of combinatorial libraries until now. Thus, an aptamer library has much higher variability than a standard drug library which dramatically increases the probability of successfully isolating sequences that bind to their targets and, if the target is a functional biomolecule, often also modulate its biological function in a cell or an organism. Furthermore, the libraries are amenable to chemical modifications that allow introduction of a broad variety of functional groups to augment their inherent propensity to recognize their targets and to improve their stability (Figure 2).[7]

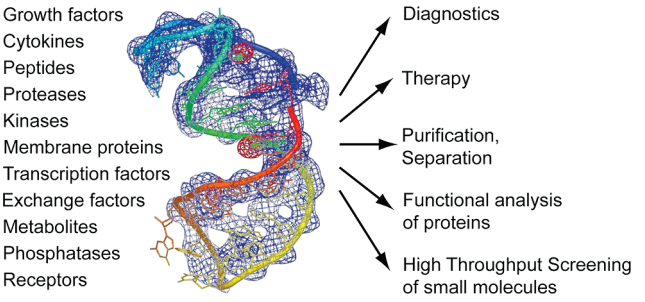

Figure 1. Aptamers that bind to a legion of different proteins and other targets find multipurpose applications in basic and applied research.

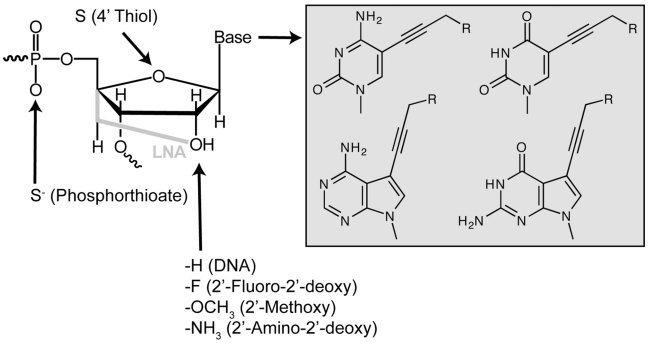

Figure 2. Summary of chemical modifications that can be introduced into aptamers. In LNA, the 2′-O and the 4′-C are bridged by a methylene group (gray line). The base modifications shown in the gray box where R comprises diverse functional groups have been shown to be compatible with DNA-SELEX replication cycles.

In 2004, the first aptamer-based drug was approved by the FDA. Pegaptanib, or Macugen, is a highly modified RNA aptamer that binds the isoform $VEGF_{165}$ of the vascular endothelial growth factor and inhibits its interaction with the VEGF receptor.[8] Pegaptanib is used for the therapy of age-related macular degeneration (AMD). Several other aptamers targeting different extracellular proteins are currently in clinical and preclinical trials for various therapeutic indications (Table 1).[9] The reason for the exclusive confinement of therapeutic aptamers to extracellular targets is that these targets represent a less demanding pharmacological

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[a] Abbreviations: SELEX, systematic evolution of ligands by exponential enrichment; FACS, fluorescence activated cell sorting; FDA, Food and Drug Administration; PCR, polymerase chain reaction; HTS, high-throughput screening; HIV-1, human immunodeficiency virus type 1; SAR, structure−activity relationship.

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Table 1. Aptamers Currently in Clinical and Preclinical Studies

aptamer	target molecule[a]	therapeutic indication[a]	reference
NOX-B11	ghrelin	adipositas	9a
E10030	PDGF	AMD	9b
NU172	thrombin	CABG	9c
ARC1779	Von Willebrand factor	ACS	9d
AS1411	nucleolin	AML	9e
REG1[b]	factor IXa	CABG	9f
Avrina	NF-κB	eczema	9g

[a] Abbreviations: PDGF, platelet-derived growth factor; AMD, age-related macula degeneration; CABG, coronary artery bypass graft surgery; ACS, acute coronary syndrome; AML, acute myelogenous leukemia. [b] REG1 comprises RB006 (aptamer) and RB007 (antidote).

challenge because they can easily be accessed in an organism without the necessity for these highly negatively charged molecules to cross membranes. Notwithstanding, it is not difficult to apply aptameric modulators of intracellular targets in cell culture. Aptamers or aptamer-encoding DNAs can be expressed in an intracellular compartement by using appropriate vectors or can be delivered directly into the cytoplasm by transfection, similar to the straightforward delivery of siRNAs. As a complement to genetic knock-down strategies by siRNAs, intracellular aptamers, or intramers, can be used for the specific recognition and inhibition of individual protein domains, subdomains, or catalytic centers of a target protein, thus providing insight into a particular epitope's role in cellular processes.[10]

While the use of aptameric modulators in cell culture is straightforward in the meantime, their application in whole organisms is hampered by their instability, bioavailability, and transmembrane delivery, at least when targeting intracellular proteins. As a consequence, it is currently difficult for me to envisage the therapeutic development of an aptamer directed against intracellular target proteins in the near future. On the other hand, the recent progress achieved in the delivery of therapeutic siRNAs or shRNAs is promising and has occurred at astonishing speed.[11] There is every reason to expect that the accomplishments in siRNA delivery systems should easily be conferrable to aptamers. Indeed, aptamer/siRNA hybrid conjugates are currently being explored as delivery vehicles for siRNAs. The concept is based on the principle that the aptamer portion of these chimeras, which binds the prostate-specific membrane antigen (PSMA),[12] undergoes cell internalization and delivers its siRNA cargo to the intracellular RNAi machinery, where the cytotoxic siRNAs then targets prostate cancer-specific prosurvival genes directly in prostate cancer cells.[13] Expanding this concept to aptamer/aptamer pairs might be a promising way to go in the future toward the development of therapeutic intramers. Nevertheless, and similar to siRNA therapeutics,[11] there still appears to be a number of obstacles that will have to be solved before the first aptamer drug directed against an intracellular target will enter into the clinic.

To cope with these limitations, we have established aptamer-displacement assays and aptamer-regulated allosteric ribozymes to "convert" the inhibitory profile of aptamers into druglike inhibitors for the same purpose. Small molecule inhibitors of proteins are invaluable tools in research and as starting points for drug development. However, their screening can be tedious, as most screening methods have to be tailored to the corresponding drug target. Thus, the idea behind this concept is that because of the ease by which aptameric inhibitors can be identified and applied to validate a target protein as "drugable", these approaches provide access to high-throughput compatible, target-independent assays for the identification of small molecules, particularly in those cases where assay development is a limiting step.[1h−1k]

During the early stage of development, we focused on aptamer-regulated allosteric ribozymes. Our goal was to construct assays, as modular and generally applicable as possible, without the need to perform chemical labeling steps of proteins, library components, or the aptamers themselves. I still remember very well when Andreas Jenne, then a graduate student in my group in Munich, approached me in excitement after he had attended a talk on real-time PCR, with the suggestion to apply a ribozyme substrate oligonucleotide labeled with a fluorophore on one end and a quencher on the other end (similar to the TaqMan probes used for real-time PCR) for the real-time kinetic analysis of ribozyme-mediated substrate cleavage. We came up with a concept based on the hammerhead ribozyme and a fluorophore/quencher-labeled RNA substrate. In these substrates a fluorophore and a fluorescence-quenching molecule lie in spatial proximity. The intramolecular fluorescence quenching is neutralized upon cleavage, and the fluorescence intensity is a measure of the ribozyme activity, allowing monitoring of the activity of ribozymes under high-throughput conditions (Figure 3).[14] Initially we had thought to apply this fully automatable reporter system in a high-throughput screening of compound libraries for inhibitors of transcription factors, as it directly measured the increase of the ribozyme concentration during a transcription reaction. We never followed up on this idea (but I think it is still a great concept that we perhaps should pick up again...). Instead, we applied the assay for the rapid identification of new ribozyme inhibitors from a small, constrained antibiotic library and a collection of 2000 bacterial extracts. Several hitherto unknown potent inhibitors of the hammerhead cleavage reaction were identified, and one of them, tuberactinomycin A, for which positive cooperativity of inhibition in vitro was found, was found to also reduce ribozyme cleavage in vivo.[15]

The next step was to expand the concept to aptamer-displacement assays, and this idea was brought rapidly forward by Jörg Hartig and Hani Najafi, two of the first-generation graduate students that worked with me after I had moved to Bonn. In the first prototype, the ribozyme activity was rendered protein-dependent by fusing its 5′-end to an aptamer to yield allosteric ribozymes, in which substrate cleavage could be triggered by the presence or absence of the protein binding partner of the aptamer. Thus, the specific disruption of the protein−aptamer complex by a small organic molecule or by another protein could also be monitored in a real-time HTS format. In this way, we identified coumermycin 1A as a new inhibitor of the HIV-1 Rev protein.[16] The principle could be modularly transferred to other aptamer−protein pairs, natural nucleic acids, and to other ribozymes, like the hairpin ribozyme.[17] For example, by applying an allosteric ribozyme that responded to HIV-1 reverse transcriptase,[18] Satoko Yamazaki found a druglike compound, SY-3E4 (**1**),[19] that bound to the aptamer binding site of HIV-1 reverse transcriptase (HIV-1-RT) rather than the active site (Figure 4). **1** inhibited the replication of HIV-1 wild-type and a multidrug-resistant strains, presumably by binding a region in HIV-1-RT that was previously unconsidered as a drugable interface. Since this new site is not targeted by the inhibitors currently available in the clinic, these findings represented

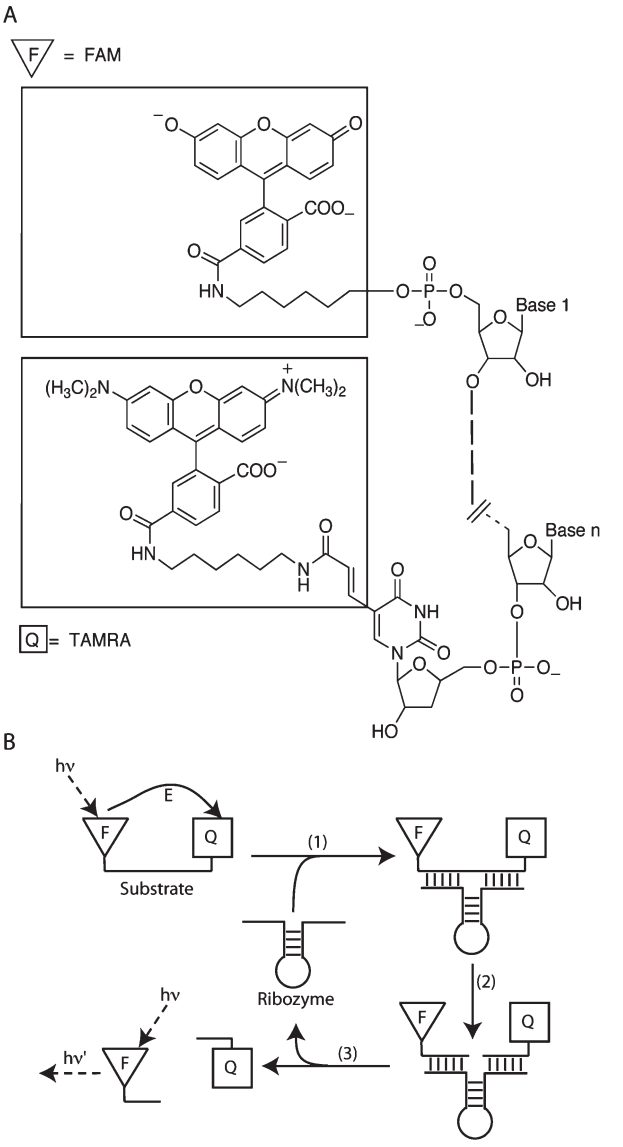


Figure 3. Schematic representation of the assay system for the real-time cleavage of the hammerhead ribozyme: (A) structure of the oligonucleotide and the fluorescence (F) and quencher (Q) groups; (B) reaction of the oligonucleotide cleavage. (1) Doubly labeled RNA substrate and the ribozyme form a catalytically active complex. (2) Doubly labeled substrate is cleaved. (3) Dissociation of the reaction products from the ribozyme renders the ribozyme available for multple turnover and results in dequenching. This leads to an increase of fluorescence ($h\nu'$) which is proportional to the rate of substrate cleavage.

a novel advance in the systematic exploration of alternative small-molecule target sites in enzymes of pathogens.[19]

More recently, we implemented aptamer-displacement screens based on fluorescence polarization by targeting a class of proteins called cytohesins.[20] Cytohesins are small (i.e., ~45 kDa) guaninenucleotide exchange factors (GEFs) that stimulate ADP ribosylation factors (ARFs), ubiquitously expressed Ras-like GTPases, which control various cellular regulatory networks ranging from vesicle trafficking to integrin activation.[21] We chose cytohesins as targets because neither knockout animals nor inhibitors were known at the time and because there are four highly related homologues in mammals that share the same domains: a Sec7-domain harboring the GEF activity, a pleckstrin homology (PH), and

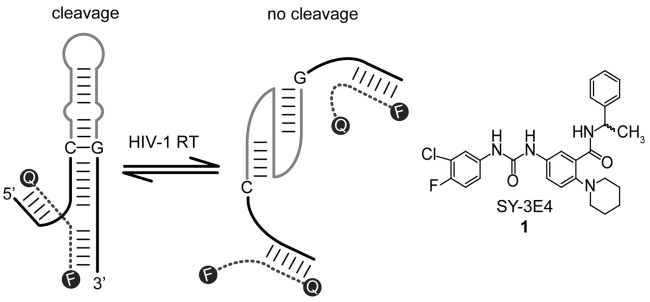


Figure 4. Schematic for the HIV-1 RT-responsive hammerhead ribozyme used in the screening assay to find inhibitors for HIV-1 RT and chemical structure of SY-3E4 (**1**). In the presence of HIV-1 RT, the aptamer sequence (gray) adopts a pseudoknot structure, disrupting the formation of stem II (left). The additional GC pair was introduced to enforce the formation of the stem−loop structure (left) in the absence of HIV-1 RT. The substrate oligonucleotide labeled with the fluorophore (F) and quencher (Q) dyes is shown as a dark-gray dotted line.

a coiled-coil domain. For the screening we used a fluorescently labeled version of a pan-selective *anti*-cytohesin aptamer termed M69 that had been identified and characterized by Günter Mayer during his Diploma thesis in Munich and Ph. D. thesis in Bonn (Figure 5).[10d] The labeling of aptamers with fluorescent tags or anchor groups amenable for screening purposes is a straightforward process that has been refined by our group and has been used for various purposes.[22]

The screening identified SecinH3 (**2**) as the first small-molecule inhibitor of cytohesins (Figure 6, left panel).[20a] SecinH3 targets the Sec7 domain of cytohesins 1, 2, and 3 and inhibits their guaninenucleotide exchange activity on ARF-proteins. When Markus Hafner and Toni Schmitz applied the pan-selective cytohesin inhibitor in human liver cells and mice, **2** allowed insulin receptor-complex-associated cytohesins to be implicated as essential for proper insulin signaling.[20a] Interestingly, while humans and mammals express up to four different but highly homologous cytohesins (cytohesins 1, 2, and 3 are found in most tissues, while the expression of cytohesin 4 appears to be restricted to cells of the immune system),[21] invertebrates like the worm *Caenorhabditis elegans* or the fly *Drosophila melanogaster* express only a single cytohesin, which however still shares very high homology to the mammalian cytohesins and also consists of essentially the same domain organization (Figure 5). Consequently, **2** not only inhibited the Sec7 domain activity of the mammalian cytohesin family members but also that of *Drosophila*. Interestingly, we found significantly reduced inhibitory activity of SecinH3 when measuring the GEF activity of members of the family of large (i.e., >100 kDa) Sec7-domain-containing GEFs such as Gea2 from yeast or the exchange factor for ARF6 (EFA6).

We also have synthesized biotinylated, photo-cross-linkable SecinH3 derivatives that can be applied as affinity probes to further determine the specificity of the inhibitor and to more precisely define the binding site of **2** on cytohesin Sec7 domains by mass spectrometry. One of these SecinH3 photoaffinity probes was obtained by integrating a benzophenone group into **2** so that its inhibitory activity was maintained.[23] Further functionalization with a detectable biotin tag led to Bio-SecinPP (**5**) (Figure 6, bottom panel), an activity-based protein profiling (ABPP) reagent for cytohesins.[23] The application of this probe in a variety of GEFs and GTPases provided cogent evidence for the specific binding of **2** to the

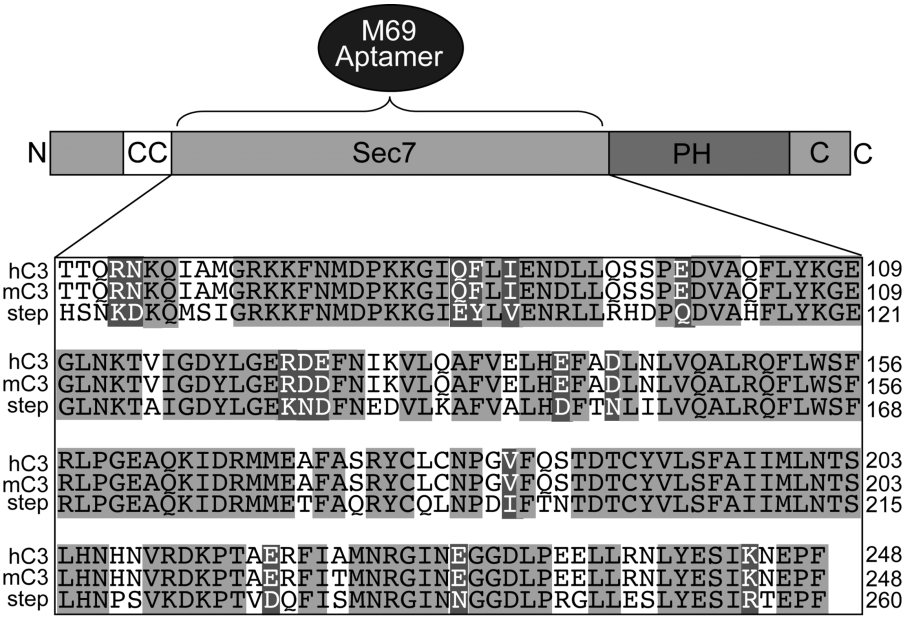
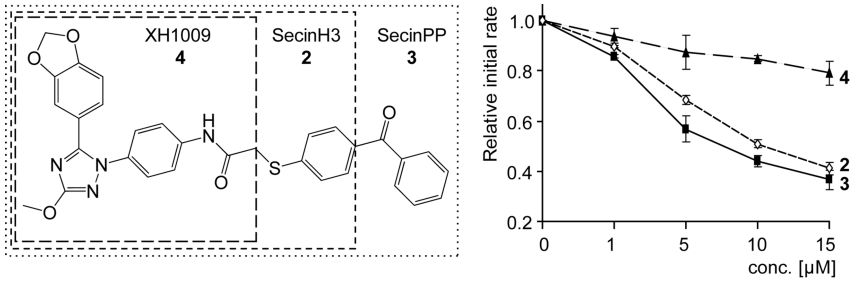


Figure 5. Domain organization of cytohesins: CC, coiled-coil domain; Sec7, Sec7 domain; PH, pleckstrin homology domain; C, C-domain. The aptamer M69 binds to the Sec7 domain of cytohesins 1−3. Sec7 domains of human and mouse cytohesins and the *Drosophila* cytohesin steppke are highly homologous. Shown are the sequences of the Sec7 domains of human cytohesin 3 (hC3), murine cytohesin 3 (mC3), and the single *Drosophila* cytohesin steppke (step). Black letters in gray background represent sequence identity, and white letters in dark-gray background represent amino acid similarity.



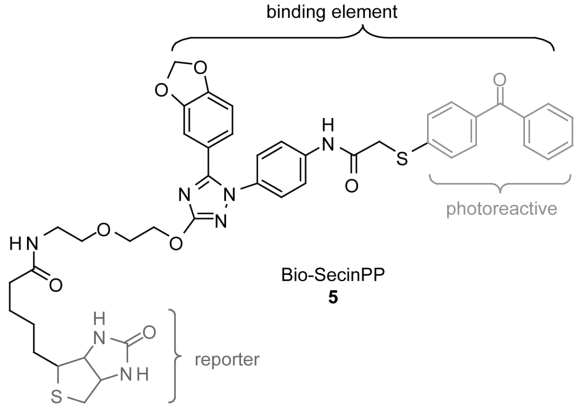


Figure 6. Chemical structures of SecinH3, the photoreactive derivative SecinPP, and the negative control compound XH1009 (left panel). Right panel: the graph shows the inhibition of cytohesin-2 (ARNO) Sec7-catalyzed guanine nucleotide exchange on [Δ17]Arf1 by SecinH3 (**2**), SecinPP (**3**), and XH1009 (**4**). Deleting the thiophenol of **2** as in **4** abolishes the inhibitory activity of **2**, whereas elongating the thiophenol as in **3** slightly increases it. Bottom panel: chemical structure of the photoaffinity probe Bio-SecinPP (**5**).

Sec7 domain of the members of the cytohesin family. We are currently synthesizing series of SecinH3 variants for SAR studies.

In parallel to our efforts to find a small molecule inhibitor for the mammalian cytohesins, my colleague Michael Hoch generated transgenic *Drosophilas* in which the single cytohesin, called "steppke", was mutated.[24] The cytohesin defect caused a phenotype similar to that seen with insulin signaling defects, namely, small overall fly size at different developmental stages but no impairment in food uptake. When wild-type flies were fed **2**, an exact phenocopy was observed. Taken together, both the cytohesin mutant flies and the SecinH3-treated flies[24]

or mice[20a] exhibited biochemical and transcriptional evidence of reduction of insulin signaling. Our chemical biology approach thus led to the discovery of a hitherto unknown component in one of the most central signaling pathways, the insulin signaling cascade, which is implicated in diseases such as diabetes or in the development of metabolic syndrome, one of the fastest-growing health problems worldwide.

In summary, there is a dazzling array of applications in biomedicine, biotechnology, and many other disciplines in which aptamers have already proven as tools of extraordinary usefulness that are easy to obtain, to handle, and to customize. In this account, I could not cover in depth every aspect of our research in which aptamers play a central role, such as our long-term program in the exploration of aptameric biosensors and arrays[25] or in nanotechnology.[26] For me, aptamer-displacement technology is perhaps the most exciting progress we have recently accomplished to bridge the two huge areas of nucleic acid biotechnology with the medicinal chemistry of small molecules. We are currently performing aptamer-displacement screens for a broad variety of targets, in particular the large protein class of guaninenucleotide exchange factors, a very important class of target proteins for which small molecule modulators are only known for a few members. The advantage of aptamer-displacement screening assays is that allosteric fluorescence labeled aptamer/target complexes can be used to explore "chemical space" highly efficiently, allowing selective domain targeting in multidomain proteins and providing screening assays if no functional assay is available. Because of the nearly unlimited variety of aptamer/target complexes, these assays are essentially target-independent and thus broadly applicable. From our published and (as yet) unpublished work it is becoming more and more evident that the small molecules that emerge from aptamer-displacement screens exhibit similar in vitro activities as their "parent" aptamers, whereas their application in model organisms like fly, mouse, or human cell culture is much more straightforward. Whatever promises the future holds for these applications, the rich and diverse implementation range of aptamers already has (at least in most cases) been a constant source of surprise and joy.

Acknowledgment. It is a great honor for me to receive the prestigious GlaxoSmithKline Award for Outstanding Achievements in the Field of Chemical Biology, and I am truly indebted to GSK for considering me a worthy recipient. I am very grateful to my scientific teachers and advisors, Prof. Dr. Gernot Boche, Prof. Dr. Julius Rebek, Jr., Prof. Dr. Jack W. Szostak, and Prof. Dr. Ernst-L. Winnacker. Although some of my co-workers were mentioned by name in the text, this is not to belittle the contributions of the many excellent students and postdocs that have worked in my laboratory over the years. Wholehearted thanks to all of you! Our work is supported by the Gottfried-Wilhelm-Leibniz program and by other grants from the Deutsche Forschungsgemeinschaft, from the Sonderforschungsbereiche SFB 624, SFB 645, and SFB 704, and from the GRK 804, the VolkswagenStiftung, the BMBF, the Alexander von Humboldt Foundation, the Fonds der Chemischen Industrie, the Minerva foundation, and the Deutscher Akademischer Austauschdienst. Last but not least, I dedicate this account to my wife Claudia for her warm-hearted, longanimous encouragement and her prudent, always reliable advice.

Biography

Michael Famulok studied Chemistry in Marburg, Germany, where he graduated in 1986 and obtained his doctoral degree in 1989. From 1989 to 1990 he was Postdoctoral Fellow at the Department of Chemistry at Massachusetts Institute of Technology. From 1990 to 1992 he was Postdoctoral Fellow at the Department of Molecular Biology at Massachusetts General Hospital. His independent career started at the Institute of Biochemistry, LMU Munich, in 1992. Since 1999 he has been Professor of Biochemistry and Chemical Biology at the LIMES Institute, University Bonn, Germany. Since 2002 he has been one of four scientific editors of the journal *Chemistry & Biology*. Michael Famulok is member of the German National Academy of Sciences "Leopoldina" and recipient of the Gottfried-Wilhelm-Leibniz Award of the Deutsche Forschungsgemeinschaft. His current research interests include aptamer technology, the chemical biology of guaninenucleotide exchange factors, and DNA nanoarchitectures.

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