Oliver Seitz

Biopolymers

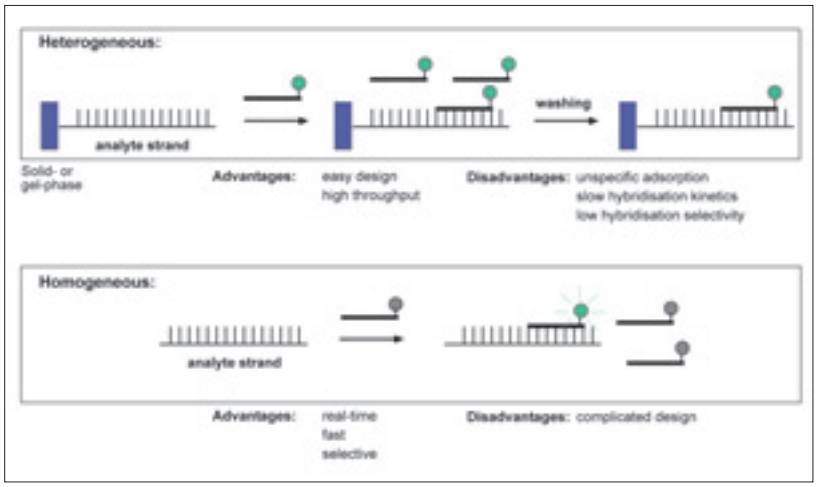
Molecular Diagnostics
Chemical Biology of Nucleic Acids

Biopolymers are the key players of life. While nucleic acids are regarded as the molecule of life the majority of life functions are mediated by another class of biopolymers, the proteins. Carbohydrates are yet another class, which constitutes a large portion of biomass. It is our aim to develop new strategies for the synthesis and modification of biopolymers in order to improve upon the existing repertoire of biomolecular tools for biological and medical research. The following chapters are intended to summarize our efforts in the field of nucleic acid chemistry and illustrate how chemistry can help in constructing tailor-made probes for the study of biological problems.

1. Molecular Diagnostics

Many diseases with different forms of appearance are genetic disorders. It is one of the chief aims of molecular diagnostics to detect a developing disease before any symptoms appear. DNA-targeted analyses play a very important role and are used in various clinical settings. The binding of a probe molecule to the complementary nucleic acid target is the molecular basis of most of the current methods in DNA-based diagnostics. In principal there are two different approaches, heterogeneous and homogeneous formats. Heterogeneous assays rely on immobilization of either the analyt or the probe molecule to a solid- or gel-phase, which facilitates the removal of unbound binding partners (Fig. 1). Areas in which binding had occurred are detectable by means of a reporter-group that is usually appended to the soluble binder. In contrast, homogeneous assays are comprised of only a solution phase and separation of unbound from bound molecules is not possible. We design new homogenous assays, which are conceptually more demanding than heterogeneous formats since the hybridization event has to be coupled with the alteration of a detectable variable. One of the advantages of homogeneous DNA-detection is that nucleic acid hybridization can be monitored in real-time even within a living cell. Furthermore, single closed-tube assays are feasible which reduces the contamination risk and speeds up DNA analysis.

Fig. 1
Heterogeneous and homogeneous DNA detection.



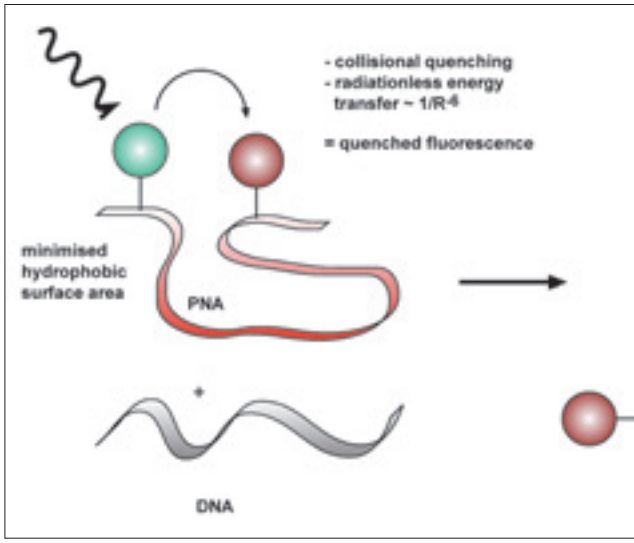


Fig. 2
Dual-labeled PNA fluorescence upon hybridization to target DNA.

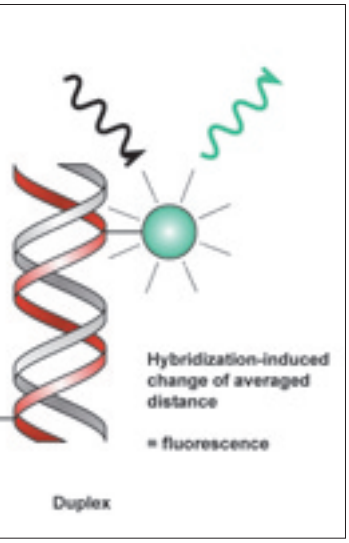
Most DNA-detection methods rely upon binding of nucleic acid probe molecules. Several oligonucleotide modifications have been developed with the aim of enhancing both the affinity and selectivity of their binding to complementary DNA and RNA. Peptide nucleic acids (PNA) are a promising class of DNA-analogues that bind with remarkably high affinity and selectivity to complementary nucleic acids (Fig. 3). A part of our research is focused on the functionalization of PNA to obtain probe molecules that meet the demands of gene diagnostic applications.

a. FIT-probes – Forced intercalation and new nucleobase surrogates for homogeneous DNA-detection

Base modified oligonucleotides are versatile probes of DNA-DNA and DNA-protein recognition. Fluorescent nucleobases and surrogates thereof were introduced in order to study localized structural alterations, which is

Biopolymere

Biopolymere sind Schlüsselsubstanzen des Lebens. Während Nucleinsäuren als Molekül des Lebens gelten, wird die Mehrheit der Lebensfunktionen durch eine andere Biopolymerklasse vermittelt, die Proteine. Kohlenhydrate konstituieren wiederum eine weitere Klasse, die einen großen Anteil an der Biomasse stellt. Es ist unser Anliegen, neue Strategien zur Synthese und Modifizierung von Biopolymeren zu entwickeln, um das existierende Repertoire an biomolekularen Werkzeugen für die biologische und medizinische Forschung zu erweitern und zu verbessern. Die vorgestellten Kapitel sollen einen zusammenfassenden Blick auf unsere Forschung auf dem Gebiet der Nucleinsäurechemie bieten und illustrieren, wie mit Hilfe chemischer Methoden Sondenmoleküle zum Studium biologischer Probleme maßgeschneidert werden.



difficult to achieve by employing spacer-linked fluorophores. Environmentally sensitive fluorophores can report binding events such as hybridization and are therefore principally suited for homogeneous DNA-detection. The fluorescence of the vast majority of base surrogates used to date becomes, however, quenched upon hybridization resulting in a less desirable negative signal. Intercalator dyes such as ethidium bromide or thiazole orange fluorescence upon intercalation between base pairs. We proposed that the use of thiazole orange as base surrogate should confer both the desirable hybridization-induced fluorescence intensification and the increased sensitivity to structural alterations as for example imposed by neighboring base mismatches (Fig. 4). [1]

In most cases, the replacement of a nucleobase by a fluorophore reduces the affinity to complementary nucleic acids. We sought to compensate potential perturbations by using peptide nucleic acid (PNA) scaffolds, DNA-analogs which are known to bind nucleic acids with high affinity. For the synthesis of the required PNA-thiazole orange conjugates we developed a strategy that allowed us to screen various intercalator dyes. It was therefore preferred to gain divergent access to PNA-dye conjugates rather than preparing a preformed monomer building block (Fig. 5). This strategy afforded probes (FIT-probes) in which hybridization is accompanied by large fluorescence increases (up to 50fold). The forced intercalation mode led to a marked sensitivity to base mismatches (fluorescence reduced by a factor of up to 16). In most cases it is even possible to distinguish a particular DNA target from its single base mutant without the need for thermal denaturation of mismatched probe-DNA complexes. This property should be of advantage in applications where stringent hybridization and wash protocols are not desired or difficult to apply such as in real-time quantitative PCR and nucleic acid detection within living cells.

b. Dual-labeled PNA for homogeneous DNA-detection

The majority of the homogenous detection methods make use of distance dependent interactions between two labels. For example, a fluorescence donor can pass its energy by a radiationless fluorescence resonance energy transfer (FRET) to an acceptor group. We explore the utility of dual-labeled PNA probes. [2, 3] It was observed that thermal denaturation of single-stranded PNA in contrast to DNA showed a phase

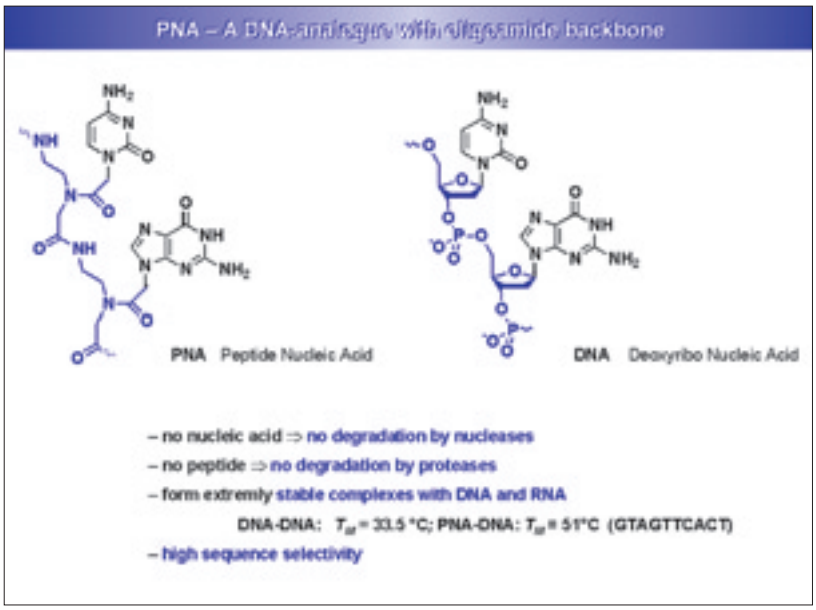
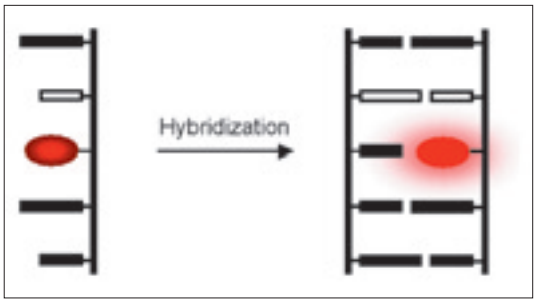


Fig. 3
DNA and the DNA-analogue PNA.

transition along with a considerable hyperchromicity, which indicated that base stacking might be a favorable process even in unhybridized PNA. A suitably appended fluorescence donor can be located in close proximity to the fluorescence quencher due to a possible inter- or intramolecular association of PNA single-strands (Fig. 2) or intramolecular collisions. As a result collisional quenching and FRET diminishes the fluorescence of the single-strand. Due to the increased flexibility of the PNA-backbone quenching should be more efficient in dual-labeled PNA than in dual-labeled DNA. Hybridization, however, induces a structural reorganization that leads to an increase of the averaged donor-quencher-distance. Thus, in the duplex,

Fig. 4
A fluorophore that serves as fluorescent base surrogate is forced to intercalate adjacent to the expected mutation site, which increases the sensitivity to the presence of mismatched base pairs (open rectangle).



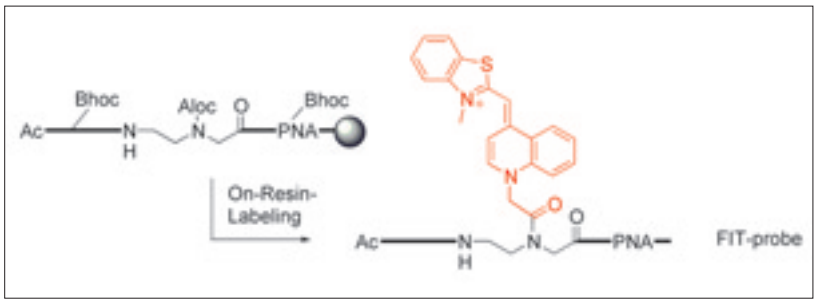


Fig. 5
The solid-phase synthesis of PNA-thiazole orange conjugates proceeds by making use of on-resin labeling techniques.

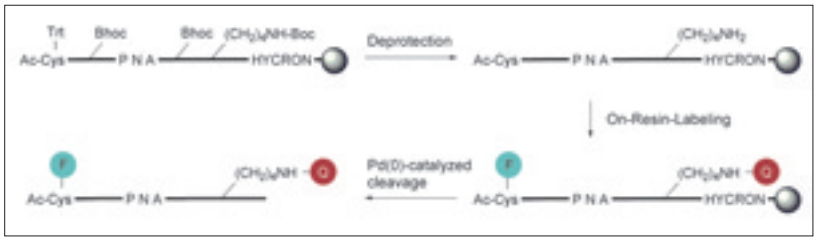
fluorescence occurs. Kinetic measurements indicated that the linear PNA probes hybridize rapidly as opposed to dual-labeled DNA probes with stem-loop structure (molecular beacons).

For the synthesis of dual-labeled PNA-probes we developed a highly flexible and automatable strategy which enabled to perform all reactions including the labeling steps on the solid phase (Fig. 6). [2, 3] A hallmark of this technique is the use of a highly orthogonal protecting group strategy in combination with chemoselective conjugation reactions.

c. Template-controlled ligation of PNA conjugates

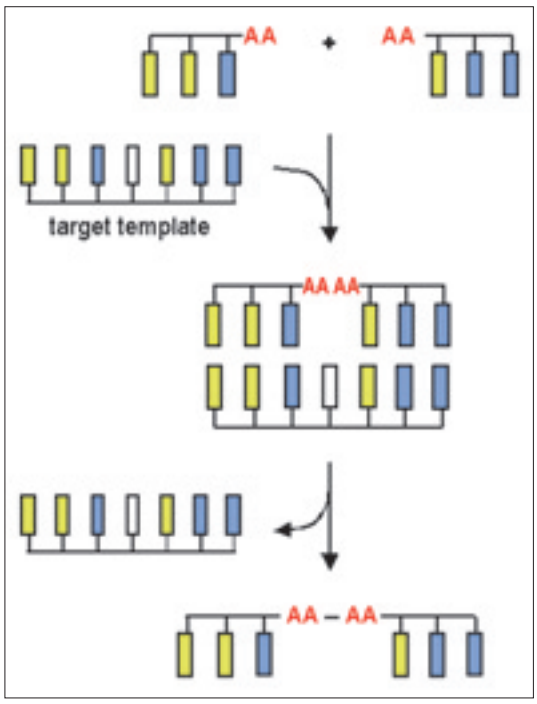
The Watson-Crick base pairing between complementary nucleotides is the universal binding mode that governs biological key processes such as replication, transcription and translation. In the terminology of chemistry, both DNA and RNA act as templates which organize substrates such that subsequent ligation reactions are facilitated. Ligation reactions that proceed under the control of a DNA-like template illustrate how chemical reactivities can be controlled by Watson-Crick base-pairing. The fidelity of such a DNA-controlled ligation reaction is a critical issue for chemical evolution and gene diagnostics and is dependent on the strength and selectivity of nucleic acid hybridization. We envisioned that a conjugation to peptide nucleic acids (PNA) enables the reacting partners of a bimolecular reaction to bind to complementary oligonucleotide-templates with high affinity and specificity.

Fig. 6
The synthesis of dual-labeled PNA succeeded by performing all reactions including the labeling reactions were performed on solid-phase.



The study of DNA-directed ligation reactions is of concern not only because of a general interest in template-directed synthesis. It is the potential regarding gene-diagnostics that intrigues us. For example, we examine whether it would be possible to design a chemical ligation reaction such that product formation would prove indicative for a certain single base mutation. Towards this end, amino acids AA are conjugated to peptide nucleic acids (PNA). In presence of a complementary oligonucleotide-template a ternary complex forms and a peptide ligation becomes accelerated (Fig. 7). The ligation strategy employed is that of a peptide coupling. In contrast to previous studies, the ligation produces a complex, in which one nucleobase remains unpaired. This was the decisive criterion which conferred a selectivity that was higher than that of a conventional DNA ligase reaction. [4]

Fig. 7
During template-controlled ligations of PNA amino acid conjugates an abasic-site is formed.



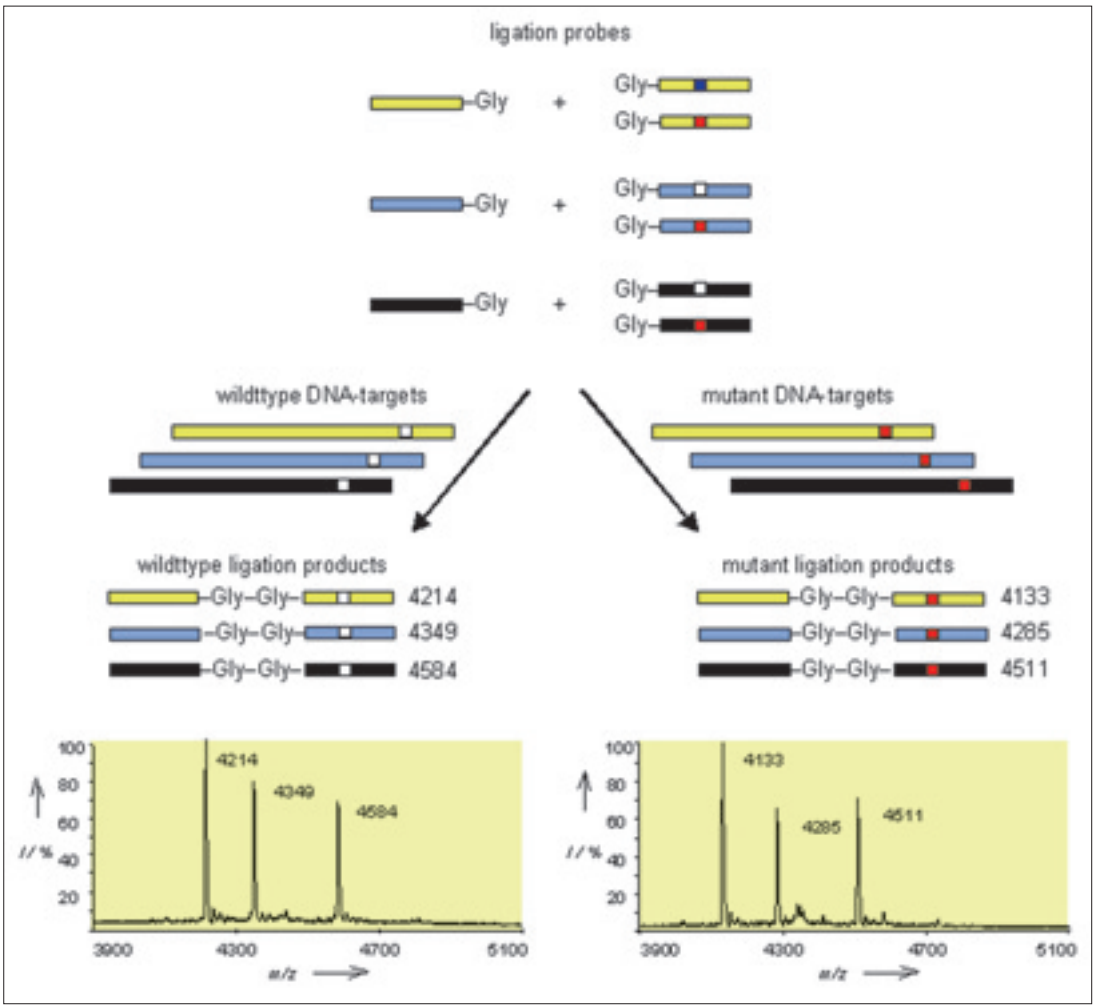


Fig. 8
Multiplexed detection of three single base mutations (white box = wildtype, red box = mutant) in three gene segments. The formation of ligation products with specified molecular masses is a marker for the presence of wildtype DNA or its single base mutants.

The high ligation fidelity enables to distinguish target DNA from its single base mutants. PNA-ligation products are easily detectable by MALDI-TOF-MS (Matrix assisted laser desorption / ionisation - time of flight - mass spectrometry). Moreover, the unparalleled resolving power of MALDI-TOF detection enables a multiplexed format to be used, thereby obtaining information about more than one mutation site within a single analysis in homogeneous solution (Fig. 8). [5]

The abasic site ligation, thus, provides new opportunities for SNP-detection. Currently we are developing a ligation chemistry for in vivo applications.

d. Ligation reactions, in which DNA acts as a catalyst

In this project we aim to devise a ligation chemistry which overcomes product inhibition and enables the DNA-template to act as a ligase. One design criterion to achieve chemical amplification is to employ reactions, in which the template-bound intermediates display a different geometry than the final ligation products.

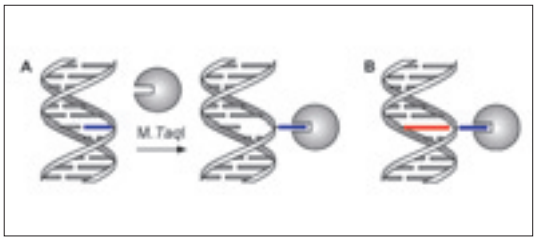


Fig. 9
Enzymatic target base (blue) flipping in A) unmodified duplexes and B) duplexes containing an aromatic base surrogate (red).

Prof. Dr. Oliver Seitz
Born 1966. Diploma in chemistry 1992 (University of Mainz), Ph.D. 1995 (supervisor Prof. Horst Kunz). After postdoctoral studies with Chi-Huey Wong at the Scripps Research Institute in La Jolla, California (1996–97) he returned to Germany (University of Karlsruhe). In 2000, he moved to the MPI of Molecular Physiology in Dortmund where he led a group in the Department of Chemical Biology and received the venia legendi for Organic Chemistry at the University of Dortmund (2002). In 2003, he was appointed as Full Professor of Organic and Bioorganic Chemistry at the Humboldt-Universität. Awards: Liebig- and Heisenberg-fellowships; Bennigsen-Foerder-Award; Lilly-Lecture-Award.
Research interests: synthesis and functionalization of biopolymers; development of new strategies for the functionalization of nucleic acids and nucleic acid analogues as tailor-made probes for molecular diagnostics.

Contact
Humboldt-Universität zu Berlin
Faculty of Mathematics and Natural Sciences I
Department of Chemistry
Brook-Taylor-Str. 2
D–12489 Berlin-Adlershof
Phone: +49–30–2093–7446
Fax: +49–30–2093–7266
E-Mail: oliver.seitz@chemie.hu-berlin.de

2. Chemical Biology of Nucleic Acids

A new concept for the inhibition of DNA-methyltransferases

DNA adopts a double-helical structure with the nucleobases buried in the interior of the double helix. For gaining steric access to innerhelical target structures DNA-modifying enzymes have evolved binding modes that lead to a local disruption of hydrogen-bonding and base-stacking interactions. For example, DNA-methyltransferases (DNA-MTases) commence the methyl group transfer by rotating the target base completely out of the helix. As a result of the enzymatic base flipping process an unpaired nucleobase remains in the duplex (Fig. 9A). In a minimum model enzyme binding has to compensate for 1) the dissociation of a Watson-Crick base pair and 2) the formation of an apparent abasic site that interrupts contiguous base-stacking. We envision that aromatic base surrogates restore the contiguous base stack, which is interrupted upon enzymatic base flipping, and thus tighten the MTase-substrate complex (Fig. 9B). In addition, polycyclic base surrogates might destabilize the innerhelical conformation of the opposing base. The DNA-MTase can thus bind to double-stranded DNA containing a preorganized unstacked target base without paying the energetic penalty for disrupting a Watson-Crick base pair and base-stacking interactions.

The incorporation of polycyclic base surrogates into duplex oligonucleotides is achieved by means of *C*-glycosidically modified nucleotide building blocks. [6] The development of a powerful *C*-glycosylation chemistry that allows the synthesis of modified phosphoamidite building blocks is hence one of the key requirements (Fig. 10). Towards this end we developed a new organocuprate-mediated *C*-glycosylation which provided improved access to nonpolar *C*-nucleosides. Binding experiments with M·*Taq*I revealed that DNA with aromatic base surrogates placed opposite to the target base binds with up to 400fold enhanced affinity. The order of binding affinities correlate well with the capability of stabilizing abasic sites in model duplexes. This suggests that polycyclic base surrogates enhance the M·*Taq*I binding affinity mainly by compensating the energetic penalty which arises from the enzyme-induced abasic site formation rather than by disrupting target base stacking.

Literature

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Members of the working group
Dr. Erika Sauer; Dr. Andrea Knoll; Dipl. Chem. (FH) Christian Dose; Dipl. Chem. Simon Ficht; Dipl. Chem. Olaf Köhler; Dipl. Chem. Lars Röglin; M. Sc. Dilip V. Jarikote.
Laboranten: Brigitte Redlich; Christina Schönherr.

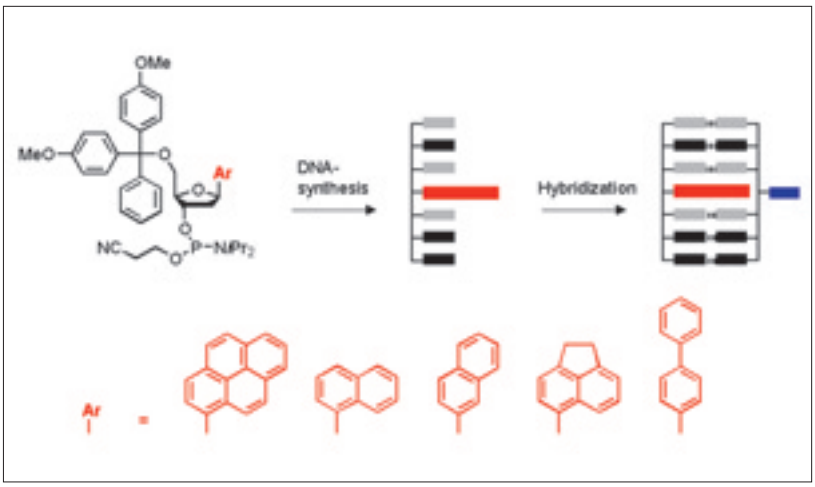

Fig. 10
Phosphoamidites containing C-glycosidically linked polycyclic aromatic base surrogates (red) are incorporated into DNA and placed opposite to target bases (blue) of DNA-modifying enzymes.