Hydrophobic effects may also be responsible for the enhanced chemical reactivity of complexes **2** and **3**. The hydrophobicity of a binding cavity can be probed by determining the binding constants of different guests.[9] For this purpose the relative stability constants (K_{rel}) of the formiato, acetato, and benzoato-bridged zinc complexes **14a–c** were determined. The stability constants increase in the order **14a** < **14b** < **14c** and differ by two orders of magnitude ($K_{rel} = 0.1/1.0/5.0$). Thus the larger the organic residue R of the carboxylate anion ($RCOO^-$) the larger the binding constant. The observed trend is indicative of hydrophobic effects between the substituents of the carboxylate ion and the ligand matrix of the complex.

The synthesis of novel binuclear complexes with hydrophobic binding pockets has been described. The complexes show enhanced chemical reactivity towards the activation and transformation of small molecules such as CO_2. We are currently probing the possibility whether the Lewis acidity can be increased by changing the metal oxidation state and whether variation of the alkyl residues allows fine tuning of the size of the binding pocket.

Received: July 5, 2001 [Z17426]

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Functionalized DNA: A New Replicable Biopolymer**

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During the past decade a number of methods have been developed that allow the isolation of biopolymers with tailored functionalities from highly diverse libraries of polypeptides or nucleic acids.[1] Polypeptide-based libraries require coding strategies such as phage-,[2] ribosome-,[3] or mRNA-display[4] that ensure an unmistakable combination of genotype and phenotype. Only then it is possible to identify a few molecules with the desired activities from mixtures of up to 10^{14} individual molecules.

In contrast, functional nucleic acids such as aptamers[5] and ribozymes[6] have the advantage that they carry along—at all times—the blueprint for their own replication, thereby making special encoding strategies for their detection and optimization obsolete. A potential disadvantage compared to polypeptides, however, is the low diversity of chemical functionalities provided by the four natural nucleotides. Expanding their functional-group repertoire will certainly further enhance their catalytic and binding properties.

Therefore, efforts currently concentrate on developing approaches that combine the advantages of the direct enzymatic amplification of nucleic acids with the chemical diversity of polypeptides by adding protein-like functionalities to the nucleobases of DNA. For example, DNA molecules containing functionalized residues were enzymatically synthesized from natural DNA templates[7] and used for in vitro selection.[8] Although the incorporation of four modified nucleotides bearing fluorescent dyes or alkynyl chains was recently reported,[9] the ultimate goal of substituting all four nucleobases with different functionalized residues within a single oligonucleotide and the subsequent use of the resulting polymer as a template for enzymatic replication has not been achieved to date. Nucleic acids that are highly functionalized in this way would represent a novel class of enzymatically replicable biopolymers that, from a chemical point of view, bridge the gap between proteins and nucleic acids, and might exhibit interesting new properties.[10]

Here we describe the first enzymatic synthesis of oligodeoxynucleotides, in which all four natural nucleobases are substituted by different synthetic residues which bring a broad range of additional chemical functionalities, and the conditions that are required for the enzymatic replication of the new oligodeoxynucleotides. Modifications were designed to include important amino acid side-chains such as carboxylic acid, alkylamino, guanidine, and hydrophobic residues. We

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[**] We thank Dr. Andreas Marx, University of Bonn, for helpful advice and discussions. This work was supported by the Fonds der Chemischen Industrie, the Karl-Ziegler Stiftung, and the Deutsche Forschungsgemeinschaft.

  1433-7851/01/4021-3990 $ 17.50+.50/0

found that the highly functionalized DNA template[11] is fully compatible with procedures commonly applied in nucleic acid in vitro selection strategies.[8a, 12]

The chemical modifications were positioned to ensure minimal interference with the face of the nucleobase involved in Watson–Crick pairing. Previous studies indicated that these requirements are best satisfied by introducing modifications at C5 in the pyrimidine bases,[7, 13] and position C7 of 7-deaza purines.[7d]

We synthesized the modified nucleoside triphosphates (dNTPs) **1**–**4**[14] (Figure 1A) and enzymatically polymerized them to a highly functionalized oligonucleotide by template-directed primer extension, using commercially available DNA polymerase from *Thermus thermophilus* (*Tth*) (Figure 1B). Using a model template with approximately equal distribution of the four nucleotides and a length of 79 nucleotides, we first optimized the conditions for primer extension with respect to nucleotide, enzyme, and template concentration, temperature, and reaction time. We found that complete primer extension of 1.0 pmol primer on 3.0 pmol template[15] (final concentration: 50 nM and 150 nM, respectively) could be achieved with only 40 μM of each modified nucleotide using 0.5 U of *Tth* polymerase per reaction at 72 °C for 2 min. Under these conditions, a single primer extension cycle led to efficient polymerization of the modified nucleotides directed by the unmodified DNA template (Figure 1B, lane 6). As negative controls, the same experiments were performed in the presence of only three modified bases omitting either **1** (lane 2), **2** (lane 3), **3** (lane 4), or **4** (lane 5), respectively. As shown in Figure 1B all four nucleotides are required to yield the full-length product. The smear that is observed in the product band that corresponds to fully extended primer (lane 6) may arise from secondary structures with different stability or higher aggregates that cannot be resolved under the conditions applied in standard denaturing polyacrylamide gel electrophoresis (PAGE).

For compatibility with in vitro selection methods in which sequence information must be reliably transferred from generation to generation it is essential to show that the enzymatically synthesized fDNA[11] can serve as a template in a polymerase chain reaction (PCR) using natural dNTPs. Thus, the fDNA was generated using a 5′-biotinylated template of unmodified DNA and the resulting fDNA/DNA hybrid was subsequently immobilized on streptavidin agarose. Unhybridized fDNA was recovered by elution with 0.1N NaOH and used as a template in a PCR in the presence of dNTPs. Under standard PCR conditions (denaturing at 95 °C, *Tth* DNA polymerase, *Tth* PCR buffer) however, no PCR product was obtained (data not shown). Since it was previously observed that the presence of modified 7-deaza purines in double stranded DNA (dsDNA) increases the dsDNA melting point[16] we reasoned that our result might be because of incomplete melting of the fDNA/DNA hybrid under the conditions applied. Therefore, we increased the melting temperature to 100 °C, used the extremely thermostable DNA polymerase from *Pyrococcus Woesei* (*Pwo*), and added "GC-Rich solution" (Roche), a commercial additive for improved amplification of GC-rich regions of dsDNA. Indeed, this combination resulted in successful amplification (Figure 2, lane 6). No amplification was obtained when the "GC-rich solution" was omitted (Figure 2, lane 5). The faint product band observed in the negative controls (biotinylated template in reaction buffer bound to streptavidin agarose, washed and eluted as described above, and

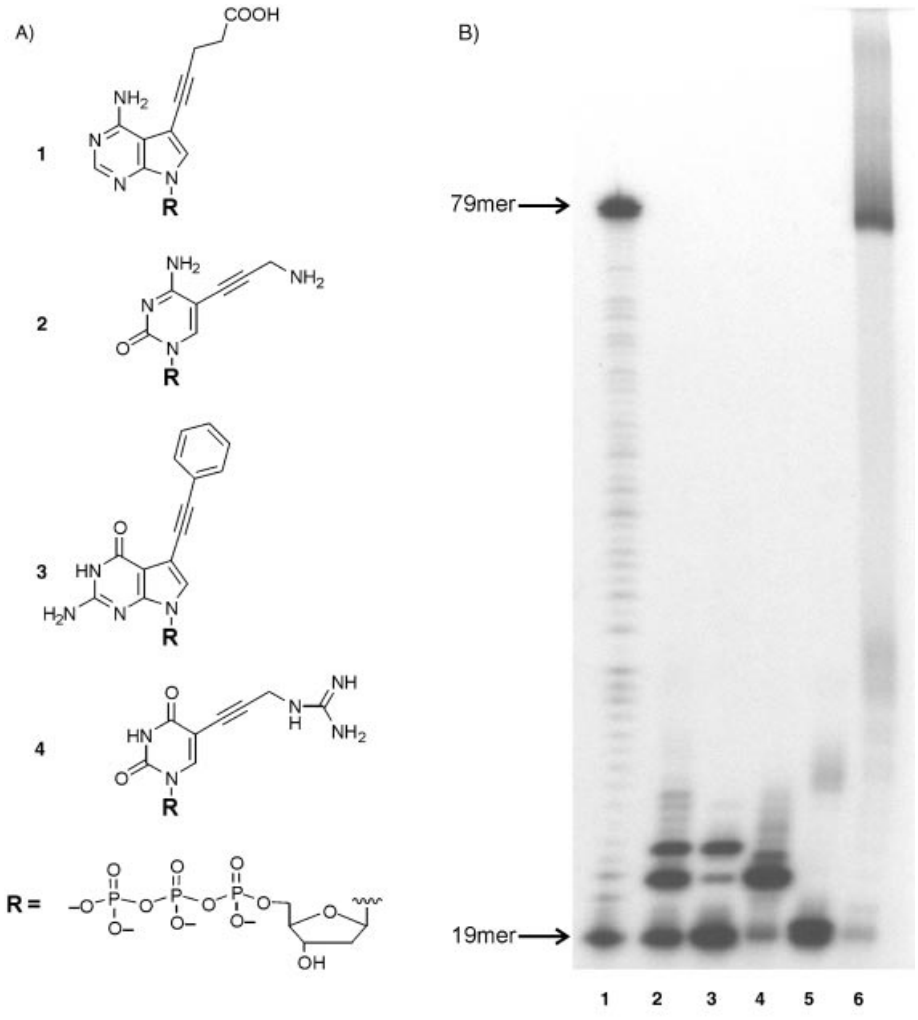

Figure 1. A) Structures of the modified deoxynucleotide triphosphates (dNTPs) **1**–**4** used in this study. Functional groups shown resemble the amino acids aspartate or glutamate (**1**), lysine (**2**), phenylalanine (**3**), and arginine (**4**). B) Autoradiogramm of an 8% denaturing PAGE of primer extension products. Lane 1: 40 μM of each natural dNTP; lane 2: 40 μM each of **2**–**4**; lane 3: 40 μM each of **1**, **3**, and **4**; lane 4: 40 μM each of **1**, **2**, and **4**; lane 5: 40 μM each of **1**–**3**; lane 6: 40 μM each of **1**–**4**.

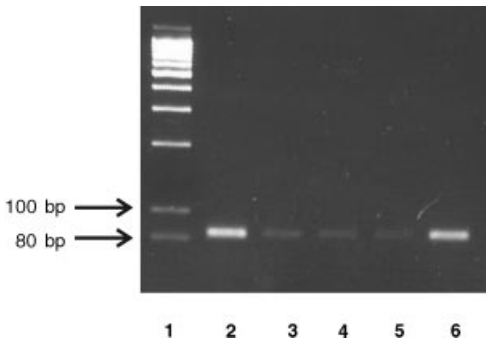

Figure 2. Ethidium bromide stained 2.5% agarose gel of PCR products. Lane 1: DNA marker; lane 2: amplification of unmodified DNA; lane 3: amplification of eluate from immobilized biotinylated template; lane 4: as lane 3 with addition of "GC-Rich solution"; lane 5: amplification of fDNA; lane 6: amplification of fDNA with addition of "GC-Rich solution"; bp = base pairs.

subjected to PCR; Figure 2, lanes 3 and 4) is a result of residual amounts of biotinylated template eluted from the agarose matrix during the treatment with 0.1N NaOH. Thus, we can define amplification conditions that are suitable for producing DNA from an fDNA template.

Finally, we purified and sequenced the DNA product that resulted from the fDNA template to check whether the sequence of the original template is retained during the enzymatic incorporation of modified dNTPs and the retransformation of fDNA into DNA. As a positive control, we used the PCR product of the unmodified DNA template. Figure 3 shows that both PCR products have identical sequences, thus verifying that incorporation of the modified nucleotides occurs sequence specifically and that fDNA can serve as a template for sequence-specific DNA amplification.

In conclusion, we present the first example of oligonucleotides with varying modifications at every single base position which can be enzymatically replicated. We demonstrated the suitability of our modified nucleotides for in vitro selection by carrying out a full cycle of primer extension and amplification by PCR. We show that nucleotides bearing amino acid like functionalities can replace natural dNTPs in primer extensions without detectable loss of sequence information. In general, functionalized DNA represents a novel class of biopolymers that bridge the gap between proteins and nucleic acids. Besides the potential application of fDNAs in combinatorial in vitro selection experiments they may exhibit interesting physico-chemical properties which will render fDNAs useful in diagnostics or functional genomics based on micro-arrays.

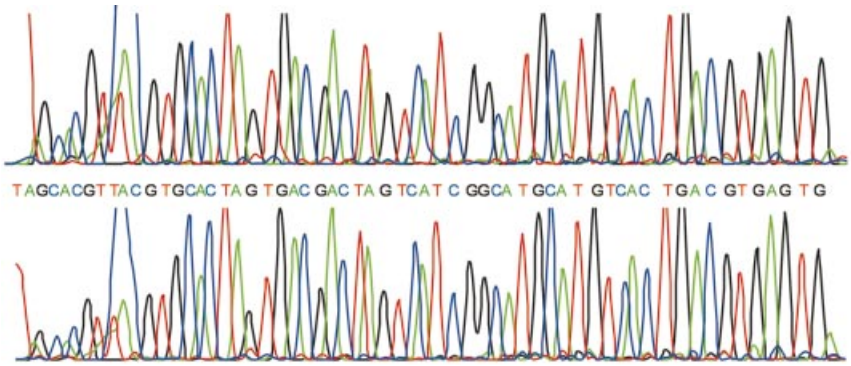

Figure 3. Cycle sequencing of PCR products using the control DNA (top) or the fDNA template (bottom). DNA sequences were obtained by cycle sequencing at PHAMISS, the DNA sequencing facility at the University of Bonn.

Experimental Section

Amplifications were performed with the 79-mer synthetic single-stranded DNA (ssDNA) template: 5′-CACTCACGTCAGTGACATGCATGCC-GATGACTAGTCGTCACTAGTGCACGTAACGTGCTAGTCAGAAA-TTTCGCACCAC-3′.

Primer extensions: An annealing mix containing 5′-[^{32}P]-labeled primer 5′-GTGGTGCGAAATTTCTGAC-3′ (1 pmol) and template (3 pmol) in reaction buffer (9.4 μL, 10 mM Tris-HCl, pH 8.9, 100 mM KCl, 1.5 mM $MgCl_2$, 50 μg mL^{-1} bovine serum albumin (BSA), 0.05% Tween 20) was heated to 95 °C for 2 min and cooled to 25 °C. *Tth* DNA polymerase (Roche, 0.1 μL, 5 U μL^{-1}), thermostable inorganic pyrophosphatase (New England Biolabs, 0.5 μL, 0.2 U μL^{-1}), and 10 μL of a reaction mix containing 80 μM of the appropriate dNTPs or modified deoxynucleotides in the same reaction buffer were added, incubated at 72 °C for 2 min and then cooled to 4 °C. Reactions were quenched by adding formamide/water (4/1, 60 μL), containing 20 mM ethylenediaminetetraacetic acid (EDTA), followed by heating to 95 °C for 10 min and immediate cooling to 0 °C. Aliquots (5 μL) of each reaction were analyzed by 8% denaturing PAGE containing 8M urea. Gels were transferred to filter paper, dried, and exposed to an X-ray film.

To separate the fDNA from the fDNA/DNA hybrid, an annealing mix containing primer (8 pmol) and biotinylated template (24 pmol) was prepared. Reactions were carried out as described above in total reaction volumes of 40 μL, using *Tth* DNA polymerase (0.2 μL; 5 U μL^{-1}) and thermostable inorganic pyrophosphatase (1 μL; 0.2 U μL^{-1}). As a negative control biotinylated template in reaction buffer without primer and nucleotides was used. All mixes were incubated at 72 °C for 10 min and then cooled to 4 °C. To each reaction buffer (10 μL: 750 mM NaCl, 0.5 mM EDTA, 250 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonioc acid), pH 7.0) was added, the mixes were immobilized on streptavidin agarose (50 μL), prewashed with wash buffer (5x100 μL: 150 mM NaCl, 0.1 mM EDTA, 50 mM HEPES, pH 7.0), followed by washing with 15 column volumes of wash buffer at 25 °C. Desired oligonucleotides were eluted with elution buffer (2x40 μL: 0.1M NaOH, 150 mM NaCl). Eluates were neutralized with 5% acetic acid (6 μL), and 10 μL were used as template for PCR amplification.

PCR (100 °C, 1 min; 50 °C, 1 min; 72 °C, 2 min, 8 cycles) was performed in 50 μL volumes, using dNTPs (200 μM), 40 pmol of each primer 5′-GTG GTG CGA AAT TTC TGA C-3′ and 5′-biotin-CAC TCA CGT CAG TGA CAT GC-3′, and *Pwo* DNA polymerase (Roche, 0.5 μL, 5 U μL^{-1}). PCR products were analyzed on a 2.5% agarose gel by staining with ethidium bromide. PCR products were purified on a preparative agarose gel, followed by desalting on a G-25 ion exchange column, and used for sequencing.

Received: May 28, 2001 [Z17184]

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Nitrogen Photofixation at Nanostructured Iron Titanate Films**

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Dedicated to Professor Günter Hauska on the occasion of his 60th birthday

Next to photosynthesis nitrogen fixation is the second most important chemical process of the biosphere. The mild reaction conditions of the enzymatic reaction compared to the Haber – Bosch synthesis stimulated a large variety of investigations on the formation and reactivity of N_2 transition metal complexes under thermal reaction conditions. Comparably little work is known on photofixation, especially in regard to a sunlight-induced nonenzymatic nitrogen fixation at a simple inorganic photocatalyst.

Schrauzer and Guth first reported that the electron – hole pairs generated by the absorption of light by a semiconductor powder can reduce molecular nitrogen to ammonia. Water vapor acted as the reducing agent, being oxidized to molecular oxygen during the process. Photoreduction occurred only when rutile-containing titanium dioxide powder was doped with 0.2 % of Fe_2O_3 and when it was exposed to humid dinitrogen. No ammonia was formed when dinitrogen was bubbled through an aqueous suspension of this powder. Higher iron contents resulted in inactive materials.[1] Subsequent work confirmed these results, although the nature of the reducing agent was unknown in most cases, since the oxygen produced was only rarely characterized.[2–14] Ammonia concentrations were in the range of 1 – 10 μM, and excitation by UV light was, in general, necessary. Very recently it was reported that an electrochemically formed titanium dioxide layer is also active without iron doping.[15] These partly contradictory results induced adverse discussions, particularly by Edwards and co-workers, and culminated in the conclusion that all the previously published values resulted from traces of the ubiquitous ammonia.[16] Since, however, it is well known that the photocatalytic properties of semiconductors are strongly influenced by the presence of impurities, the contradictary results may stem from difficulties in preparing the catalyst.

To clarify these adverse results we have prepared mixed iron titanium oxides by a simple and highly reproducible sol – gel method. In contrast to the previously employed titanium dioxide photocatalysts, the new materials were applied as nanostructured thin films containing up to 50 % iron. They also photocatalyze the formation of ammonia and nitrate under visible light.

The films were obtained by immersing a glass slide first into an alcoholic solution of iron(III) chloride and titanium tetraisopropylate (1:1), followed by hydrolysis in humid air and annealing at 600 °C; only an inactive film was produced at 500 °C. The iron-free titanium dioxide film was prepared in an analogous manner. Electron microscopy on the iron titanate film indicated the presence of a nanostructured matrix of about 300 nm thickness. It contains 15 – 20 vol % of cubic crystals with an average diameter of 150 nm (Figure 1). The ratio of Fe:Ti:O was found by energy-dispesive X-ray spectroscopy (EDAX) to be 1:1:3.5 for both the matrix and the crystals. This composition suggests that the compound $Fe_2Ti_2O_7$ is present, which was previously only obtained as an intermediary phase by heating ilmenite ($FeTiO_3$) minerals in an oxygen atmosphere to 700 °C.[17] This assignment is corroborated by the good agreement between the published and measured X-ray diffraction (XRD) spectra. The doublet at $\delta = 0.462\ mm\,s^{-1}$ (relative to α-Fe), $\Delta E_Q = 0.910\ mm\,s^{-1}$, line widths: $0.294\ mm\,s^{-1}$)[18] in the Moessbauer spectrum points to the presence of a six-coordinated Fe^{III} ion. The UV/Vis spectrum reveals extended absorbance down to 800 nm (Figure 2).

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[**] This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. Experimental help by Dipl.-Chem. Harald Weiß is gratefully acknowledged.

  1433-7851/01/4021-3993 $ 17.50+.50/0