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The Large Fragment of *Escherichia coli* DNA Polymerase I Can Synthesize DNA Exclusively from Fluorescently Labeled Nucleotides

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Fluorescence is a highly specific and sensitive property that is frequently used for detecting and identifying certain molecules within synthetic or biological materials. Particularly the augmentation of traditional Sanger sequencing with fluorescent primers or terminators paved the way for the most durable and efficient method of DNA sequencing. With the rapid progress in fluorescence technology, and encouraged by the international race to sequence the human genome as well as the genomes of other model organisms, the idea to combine fluorescent sequencing with single-molecule detection became obvious, and it nourished the hope to simplify and speed up the task of sequencing DNA segments as long as 50 000 bp and joining the sequence information from genome fragments.[1–4] However, single-molecule sequencing requires the complete and faithful tagging of each of the four types of bases of DNA (or RNA) with its own fluorescent compound. In principle, the labeling may be achieved by DNA-polymerase-mediated incorporation of fluorophore-labeled deoxynucleoside triphosphates. In practice, most natural DNA polymerases have been found to discriminate against bulky fluorescent nucleotide analogues which often also exhibit a net charge differing from that of the natural substrates.[5–8] As yet, only few mutant bacteriophage T4 DNA polymerases have been identified that exhibit an increased capability of incorporating modified monomers for the synthesis of long chains of complementary fluorophore-labeled DNA.[9]

We employed the well-documented power of directed evolution[10] to get access to one or more DNA polymerases with the abilities to 1) incorporate a fluorophore-labeled nucleotide, 2) extend the terminus by addition of the next fluorophore-labeled substrate, and 3) retain a sufficient incorporation fidelity. Therefore, we developed a functional screening system that allowed for the assessment of individual clones that show an increased acceptance of fluorophore-labeled nucleotides, and we started the search for the desired enzymatic activity by using the Klenow fragment (KF) of DNA polymerase I (Pol I) of *Escherichia coli*.[11–15] Because elongation of a primer–template is not a single-step reaction requiring simple yes/no decisions, we employed fluorescence correlation spectroscopy (FCS), a highly sensitive method that enables the distinction of chain lengths and fluorescence intensities of individual nucleic acid molecules.[16,17]

The set-up of a fluorescence-based assay for screening active variants within DNA polymerase libraries then revealed that, surprisingly, the wild-type KF retains full activity in the sole presence of artificial deoxynucleoside triphosphates that are labeled with a fluorescent dye of the rhodamine type. The screening procedure that allowed for this discovery included 1) the cloning, individualization, and overexpression of functional KF or its variants, 2) the immobilization and purification of polymerase protein, 3) the polymerase reaction, 4) the immobilization and purification of reaction products, and 5) the spectroscopic distinction of educts and dye-labeled DNA products according to their diffusion times and fluorescence intensities (Figure 1).

The comparative evaluation of individual polymerase variants for activity with unnatural substrates required two purification steps: On the one hand, polymerase mutants that were cloned and expressed in *Escherichia coli* had to be separated from all competing host DNA polymerases, and on the other hand, reaction products, possibly of varying lengths, had to be purified from an excess of fluorescent monomer which could interfere during the FCS measurement. Because extensive purification steps drastically reduce the number of individual mutants that can be tested in a certain period of time (throughput), we employed one-step procedures for solving both problems, and we applied commercially available microwell formats.

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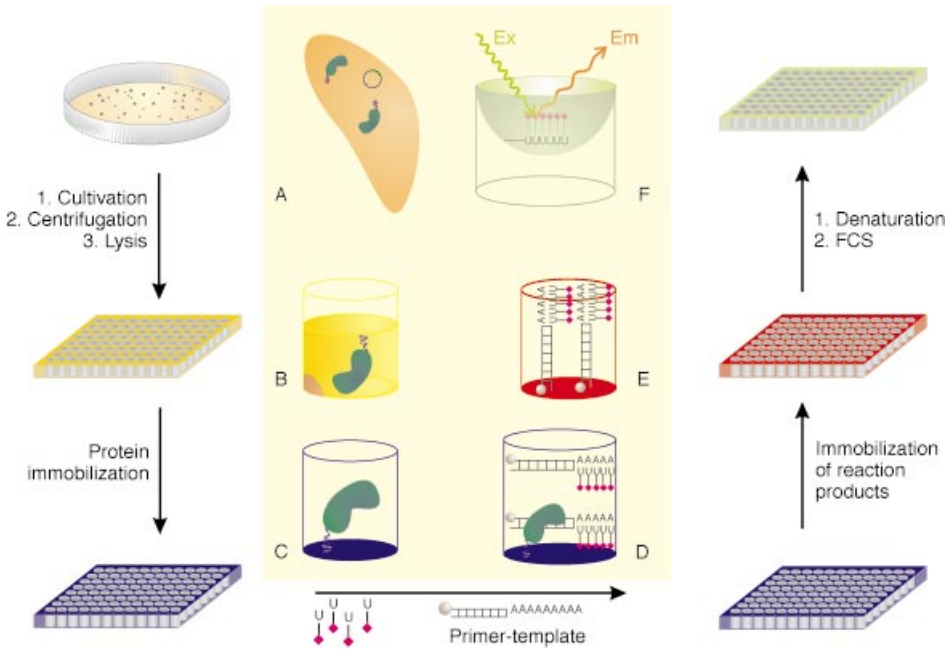

Figure 1. *Principle of the DNA polymerase assay. Clones expressing six-histidine-tagged KF or its mutant derivatives (A) are transferred into a microwell plate for parallel cultivation (B). Following lysis and separation of cell debris, soluble proteins are transferred to a Ni-NTA-coated (NTA = nitrilotriacetic acid) microwell plate for binding and purification of His-tagged protein (C). The reaction to be assayed is started by adding biotinylated primer – template and fluorescently labeled deoxynucleoside triphosphates to the immobilized polymerase which is resuspended in reaction buffer (D). Biotinylated products are bound to the streptavidin coating of another microwell plate and purified from excess fluorescent substrates and other contaminants (E). Labeled DNA products are liberated from streptavidin and transferred to a microwell plate suitable for FCS analysis (F). Interpretation of the data reveals the diffusion times and fluorescence intensities of labeled products.*

Regarding a simplified protein purification procedure, we generated a six-histidine-tagged polymerase expression construct by ligating the wild-type KF-encoding gene (source: *E. coli*) into an expression vector that encoded the N-terminal version of this tag (pQE30; Qiagen). Strain XL2Blue (*recA1*; Stratagene) that was transformed with this construct (pQKlenow) expressed high levels of recombinant KF protein which exhibited the expected high affinity to nickel-nitrilotriacetate-(Ni-NTA)-coated matrices and, thus, allowed for a one-step purification from contaminating proteins (typical yield of purified KF: 4 – 7 μg mL^{-1} culture). Furthermore, the purified enzyme was shown to retain its polymerase activity while immobilized on the affinity material.

Because the 3′ →5′-exonuclease function of wild-type KF was suspected to interfere with the intended screening for polymerase activity with unnatural substrates, we eliminated the respective function by introducing two amino acid replacements, D355 →A and E357 →A. In accordance with results obtained by Derbyshire et al.,[18] site-directed mutagenesis yielded a KF derivative with full polymerase activity in the complete absence of exonuclease activity and, thus, enabled us to focus on a single enzymatic function. Randomization of this genotype (pQKlenowExo$^-$) was then achieved by PCR-based mutagenesis[19] and resulted in the mutant library pQKlenowExo$^-$Mut.

Referring to the assay reaction, we designed the 5′-biotinylated template Rho-58 which allowed for the binding of biotinylated, double-stranded product DNAs to streptavidin-coated microwell plates (Dunn) and, therefore, enabled the efficient removal of excess fluorescent monomer and other contaminants by simple washing. Elongation of the appropriate primer – template required polymerization along a homopolymeric $(dA)_{58}$ stretch of the template strand. This type of selective constraint was thought to force the multiple successive incorporation of fluorescently labeled deoxynucleoside triphosphates because steric as well as electronic hindrances were viewed as the major obstacles of the interaction between polymerase, primer – template, and dye-labeled substrates. As yet, the maximum chain length of directly adjacent fluorescent deoxynucleoside triphosphates that were incorporated enzymatically has been documented for up to seven cyanine-labeled deoxyuridylic acid residues.[20]

We performed the polymerase assay with exonuclease-deficient KF that was bound to the wells of Ni-NTA-coated microwell plates. After addition of template Rho-58 that was previously annealed to the appropriate primer (S3), as well as tetramethylrhodamine-4-dUTP (TMR-dUTP, FluoroRed; Amersham Pharmacia) as the sole deoxynucleoside triphosphate as substrate, elongation of the primer – template was achieved under standard conditions. Reaction products were purified by employing streptavidin-coated microplates, liberated from the affinity material, and subsequently submitted to FCS analysis. Interpretation of the FCS data (autocorrelation curves) revealed the prevalence of a single molecular species which exhibited an average diffusion time of 306 μs, that is, twofold slower than the diffusion time of 152 μs exhibited by the substrate TMR-dUTP. Instead of multiples of the number of photoelectrons observed with TMR-dUTP, however, the values that were obtained with Rho-58 reached a maximum of 85 % of the monomer intensity. This finding can be explained with the high density of rhodamine dyes that are attached to adjacent bases through flexible linkers: It is known that especially guanosine (present within the primer-binding sequence of Rho-58/S3) effects a substantial quenching of rhodamine fluorescence.[21,22] Similarly, dye – dye interactions of the planar rhodamine dyes, which will be favored at higher labeling densities, most probably also promote the quenching of fluorescence (see Supporting Information for details).

The Rho-58 product samples were also submitted to electrophoretic analysis on 12 % (w/v) polyacrylamide sequencing gels. Resolution of the fluorescent products in an automatic system (ABI 373 A; Applied Biosystems) allowed for a quantitative determination of fragment lengths as well as an estimate of the product yield (GeneScan 672 software; ABI). The lengths of

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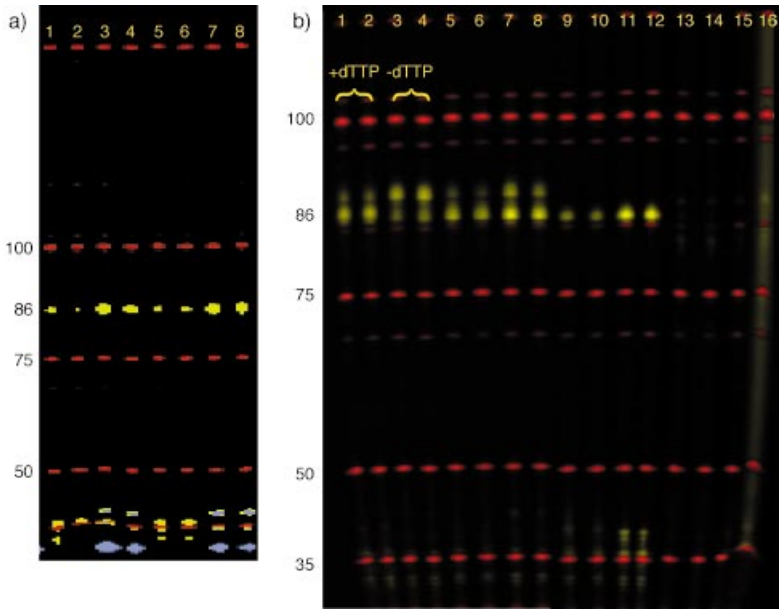

Figure 2. a) *Quantitative product analysis after separation of Rho-58 product(s) on denaturing 12% (w/v) polyacrylamide gels and resolution by using the ABI 373A system and GeneScan 672 software (internal marker: ROX-350; expected product length: 87 nt). Lanes 1 and 2 show the product produced by wild-type KF, lanes 3 and 4 show the product obtained with primer S3 that was previously 5′-cross-labeled with the dye Rhodamine-Green, lanes 5 and 6 represent the product yielded by exonuclease-deficient KF, and lanes 7 and 8 show the respective cross-labeled product. b) Analogous analysis of products produced by other DNA polymerases using either a 1:1 mixture of dTTP and TMR-dUTP, or TMR-dUTP only. Lanes 1 – 4 show the result produced by Vent Exo$^-$, lanes 5 – 8 represent DeepVent Exo$^-$, lanes 9 – 12 show KF Exo$^-$, and lanes 13 – 16 represent Tgo Exo$^-$.*

products that resulted after elongation of primer – template Rho-58/S3 with TMR-dUTP was determined to be 86 nucleotides (nt), that is, 1 nt shorter than expected on the basis of the respective template length (Figure 2a).

Thus, both analytic approaches, FCS as well as quantitative fragment length determination in sequencing gels, suggested that the exonuclease-deficient KF is capable of catalyzing the template-instructed polymerization of 58 tetramethylrhodamine-labeled deoxyuridylic acid residues. This finding was quite surprising because 58 basepairs were expected to build up more than five turns of the DNA double helix, which should receive immense steric and electronic constraints due to the bulky rhodamine dyes. In order to test whether this hitherto unknown DNA polymerase activity was due to the inactivation of the exonuclease function, we repeated the experiment with wild-type KF under analogous conditions. Again, FCS analysis as well as electrophoretic separation followed by fragment length determination revealed the predominance of full-length dye-labeled product. Most interestingly, among other DNA polymerases that were tested under the same experimental conditions, Vent Exo$^-$ as well as DeepVent Exo$^-$ (both from New England Biolabs) produced a similar product together with a by-product, whereas Tgo Exo$^-$[23] yielded no product at all with the artificial primer – template Rho-58/S3 and TMR-dUTP as substrate (Figure 2b).

Considering the electrophoresis of DNA in general, a linear reciprocal relationship exists between the number of base pairs of DNA (or, its size) and its mobility.[24] Because second-strand synthesis of primer – template Rho-58/S3 with 58 TMR-labeled deoxynucleoside triphosphates increased the molecular weight of dye-labeled product by a factor of ca. 1.4, we wondered whether the electrophoretic mobility of extensively dye-labeled DNA was still linked to the fragment length by a linear relationship. Therefore, we verified the correct product length determination by submitting a series of incrementally shortened Rho-*n* templates ($n = 2$, 4, 8, 12, 16, 20, 30, 40) to analogous labeling reactions, and by resolving the purified products in sequencing gels. Figure 3 shows that, in contrast to our expectations, the mobility behavior remains almost unchanged, and that the calculated fragment lengths deviate by a consistent value of 1 – 2 nt only. Thus, we can conclude that the fragment length analysis enabled a reliable product identification.

Interestingly, we did not find a linear relationship between fragment lengths and the respective diffusion times measured by FCS (data not shown). This fact may be attributed to the nondenaturing FCS conditions which do not hinder intramolecular interactions (dye – dye or dye – nucleotide interactions), and which thus may support deviations from the common linear shape of double-stranded DNA.

The screening system described here provides for the assessment of polymerase activity in the sole presence of unnatural, fluorescently labeled deoxynucleoside triphosphates, and it 1) may be applied to other

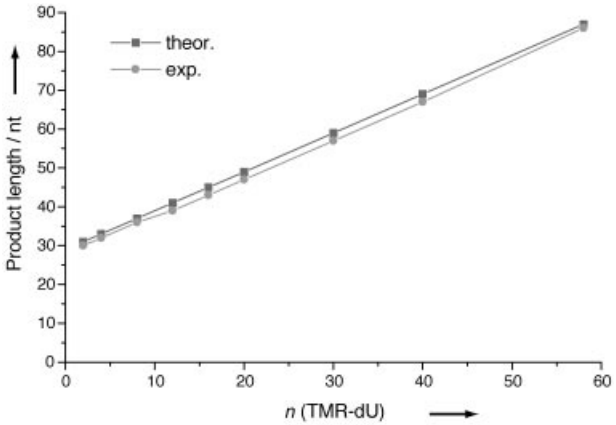

Figure 3. *Calibration of product analysis by using 12% (w/v) polyacrylamide sequencing gels.*

enzymes that catalyze the stepwise polymerization or, likewise, the stepwise degradation of nucleic acids, and 2) will be suitable for automation. The FCS-based assay allowed for a highly efficient and highly sensitive detection of product formation even in the femtomolar concentration range and, additionally, was shown to yield highly reliable diffusion time values that differed by a maximum of ca. 10% for identical clones (data not shown).

It is, of course, fascinating to elucidate why a growing primer – template, which already harbors some bulky rhodamines, may

be accomodated and even elongated with additional labeled nucleotides by a DNA polymerase that has emerged during natural evolution as an enzyme "fit" for natural substrates. Although there is as yet no high-resolution structure of a complex between KF and a primer–template bound in the polymerase active site, useful insights into the enzyme's contacts to DNA may be provided by a structure of an editing complex of KF and the duplex portion of DNA having a 3′ overhang.[25] This structure shows two deep clefts, one of which is located in the polymerase domain that shows a cluster of residues which are conserved between KF and bacteriophage T7 DNA polymerase (T7 DNAP).[26] The recently solved structure of T7 DNAP also revealed that the enzyme's active site forms contacts to the bases through numerous van der Waals interactions,[27] and, therefore, can precisely recognize the geometry of each base pair.[28] If the same was true for KF, its catalytic cleft should show considerable flexibility to surround a "swollen" DNA structure. Furthermore, experimental evidence exists that duplex DNA with one completely labeled strand undergoes a transition from a right-handed helix (B-DNA) to its left-handed form (Z-DNA).[29] This suggests that the polymerase active site of KF also does not discriminate against one of these two forms of duplex DNA.

Complete labeling of each base within one strand of duplex DNA with a distinctly coding fluorescent dye is a necessary prerequisite for single-molecule sequencing. The screening approach described here represents a promising strategy for detecting the required polymerase activity in the sole presence of a variety of artificial substrates—as long as they emit fluorescence. Furthermore, our finding of KF as an ideal enzyme for polymerizing artificial homopolymeric DNA exclusively from rhodamine-labeled monomers opens up the horizon for generating heteropolymeric DNA templates ready for sequencing that are completely labeled with (distinctly coding) dyes of the rhodamine type. Beyond that, it will be enlightening to solve the structural basis for this unexpected polymerase function.

Experimental Section

Cloning, expression, and immobilization of functional KF or KF mutant libraries: The 1.8-kb fragment coding for KF was PCR-amplified starting directly from genomic DNA of *E. coli* by using the primer pair 5′-TCG ATC GAT CGA CCA GGA TCC ACG GCA ACG *GTT*-ATT TCT TAT GAC-3′ (EcoI 3.1) and 5′-TCG ATC GAT CGA AAG G-CAT CT GCA GGC GAA TCT TAG TGC G-3′ (EcoI 4; restriction sites underlined; modified start codon in italics). The PCR-generated wild-type fragment was ligated into expression vector pQE30 (coding for an N-terminal six-histidine tag; Qiagen) yielding plasmid pQKlenow (wild-type KF gene). Removal of the 3′ →5′ exonuclease activity was achieved by using primer 5′-GGC TGT CGG TTG CGG TAG CAA ATG-CAA ATA CCG GCG G-3′ together with primers EcoI 3.1 and EcoI 4 for the one-tube PCR-based substitution of amino acids Asp 355 (codon GAT) and Glu 357 (codon GAA) by Ala (codons GCT, or GCA, respectively; minus-strand sites underlined) according to Picard et al.[30] Subsequent ligation of the mutated gene into pQE30 yielded plasmid pQKlenowExo$^-$. Randomization of the gene encoding exonuclease-deficient KF was achieved by using the primer pair EcoI 3.1/EcoI 4 in a protocol for error-prone PCR that was developed by Cadwell and Joyce.[19] The resulting mutant library was ligated into pQE30 yielding pQKlenowExo$^-$Mut.

Expression of wild-type or mutant polymerase was achieved after transformation of strain XL2Blue (*recA1*, effecting reduced homologous recombination of DNA; Stratagene) with a KF expression plasmid (*tac* promoter). Individual clones were transferred from agar plates into a 96-well plate (Deep Well Plates; Qiagen) containing 500 μL Luria–Bertani (LB) medium per well that was supplemented with ampicillin in standard concentrations. After sealing the 96-well plates with Air Pore Tape sheets (Qiagen), the cultures were shaken for 19 h at 37 °C. These overnight cultures were diluted tenfold into fresh medium (1.5 mL per well) and allowed to incubate until cells were grown to the optical density at 600 nm (OD_{600}) of 0.7–0.9 (spot-checked). Protein expression was induced by the addition of 30 μL isopropyl-β-D-thiogalactopyranoside (IPTG; 50 mM) to each well, and incubation was continued for 2.5 h. Cells were harvested by centrifugation (microplate buckets; Megafuge 1.0; Heraeus), resuspended in lysis buffer (50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 1 mM dithiothreitol, 1.5 mg mL^{-1} lysozyme) and allowed to incubate during 45 min shaking at 4 °C. Cell debris was separated by another centrifugation step leaving all soluble proteins in the supernatant. Aliquots of 150 μL of the lysate—typically containing 6–10 μg KF—were transferred into fresh microwell plates coated with nickel-nitrilotriacetate (Ni-NTA plates; Qiagen), mixed with 50 μL binding buffer (200 mM K-phosphate, pH 7.2, 600 mM NaCl, 0.8% bovine serum albumine (BSA)), and shaken for 20 h at 4 °C. After decanting of the supernatant, unbound protein was removed by washing three times with 200 μL washing buffer I (50 mM K-phosphate, pH 7.2, 150 mM NaCl).

Templates: All synthetic templates for the polymerase assay as well as for the calibration experiments were of the type 5′-biotin-A_n-GGA TGT GGG TGA TGC ATA TTT TTC AGT-3′ (termed Rho-*n*; $n = 2, 4, 8, 12, 16, 20, 30, 40, 55, 58$). Primer–template for a single reaction (one well) was prepared by mixing 1.1 pmol primer 5′-(GA) ACT GAA AAA-TAT GCA TCA CCC ACA TCC-3′ (termed S3) with 1 pmol of the respective template, heating the mixture for 2 min to 100 °C, and cooling to 20 °C within 1 h.

Assay of polymerase activity: Elongation of primer–templates with immobilized polymerase was performed in 50 μL reaction buffer (10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 12.5 mM dithiothreitol) containing 25 mM tetramethylrhodamine-4-dUTP (FluoroRed; Amersham Pharmacia Biotech) as the sole deoxynucleoside triphosphate. Reactions were allowed to proceed during 30 min of shaking at 37 °C and subsequently transferred to streptavidin-coated microplates (Dunn) that had been rehydrated according to the manufacturer's instructions. After addition of 50 μL 2 × SSC buffer (0.3 M NaCl, 0.03 M Na-citrate), immobilization was achieved during 1 h of incubation at 37 °C. Immobilized reaction products were washed with 300 μL washing buffer II (0.1 M Na-phosphate, pH 7.2, 150 mM NaCl) three times. A final wash step with 100 μL 90% formamide during 15 min at 60 °C liberated the fluorophore-labeled DNA, which then was diluted tenfold with water. 35 μL of this solution were transferred to a fresh microplate (Polyfiltronics) and monitored with FCS.

Product analysis: Fluorescence correlation spectroscopy: The experimental set-up was derived from a standard FCS apparatus.[31] A detailed description can be found in the Supporting Information.

Quantitative fragment analysis: Electrophoretic separation of randomly chosen product samples was achieved by using sequencing gels (12% (w/v) polyacrylamide, 8.3 M urea, internal fragment length standards ROX-350 or ROX-1000; ABI) with resolution on an ABI 373 A sequencer. Fragment lengths and relative concentrations were determined with the GeneScan 672 software (ABI).

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Dependence of Concanavalin A Binding on Anomeric Configuration, Linkage Type, and Ligand Multiplicity for Thiourea-Bridged Mannopyranosyl – β-Cyclodextrin Conjugates

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KEYWORDS:

carbohydrates · concanavalin A · cyclodextrins · glycoconjugates · lectins

Concanavalin A (Con A), the mannose-specific lectin from *Concanavalia ensiformis*, has long been used as a model for carbohydrate – protein interactions.[1] Its commercial availability and the rather extensive structural knowledge currently available make it attractive for assessing and optimizing the functional parameters that affect its affinity for mannose neoglycoconjugates. Understanding these key elements may facilitate the development of new therapeutic strategies based on specific recognition events such as targeting of drugs.

Con A binds α-D-mannopyranosides preferentially over the corresponding β anomers.[2] The affinity for monosaccharide ligands is low; however, this is a rather common feature when considering protein – carbohydrate interactions. Carbohydrate – protein binding events usually involve several simultaneous contacts between carbohydrates that are clustered on cell surfaces and protein receptors that contain multiple carbohydrate-binding sites. Based on this concept, one could anticipate that multiplication of the saccharide epitope on the surface of the carrier may lead to a greater affinity than predicted from the sum of the constitutive one-to-one interactions[3, 4]—the so-

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*Supporting information for this article (physical and spectroscopic data for compounds **5**, **7**, **9**, **16**, **17**) is available on the WWW under http://www.chembiochem.com or from the author.*