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PAPER

Cyclodextrin - peptide conjugates for sequence specific DNA binding

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Synthetic models of bZIP transcription factors have been developed with the capability of specific DNA recognition. Our design is based on the CuAAC mediated conjugation of basic region Leucine Zipper peptides to different derivatives of α, β and γ-cyclodextrins equipped with azide functionalities. Thorough optimization of reaction conditions allowed convergent and simultaneous conjugation of two long unprotected cationic peptides to cyclodextrin-bis azide derivatives. The resulting constructs were shown to specifically recognize their cognate DNA sequence with nM affinities. In comparison with previously developed TF models the here described derivatives combine enhanced DNA binding capabilities with an easy and convergent synthetic route.

Introduction

The study of gene expression regulation is currently of particular interest due to the upcoming development of gene therapy strategies. Indeed, altered expression of particular genes can cause interferences with biological processes in the cell. Therefore, selective up- or down-regulation of specific gene transcription could ultimately result in a therapeutic platform with biomedical applications. During the past decade, biologists and medicinal chemists have been working together in order to target specific genes for this purpose[1–3].

In this context, Transcription Factors (TFs) have been studied in great detail for the modification of gene expression and the study of DNA binding affinity in the cellular environment[4–7]. In particular, bZIP proteins have gained attention due to the simple arrangement of the protein structure consisting of a well-defined dimerization domain and a basic binding region[8]. These proteins are able to interact with DNA in a sequence-specific manner by means of inserting their recognition domains, consisting of defined sequences of amino acids, in the DNA major groove. Simplification of such proteins could ultimately result in peptide-based drugs for alternative disease treatment. Moreover, due to the improvements in peptide manufacturing, peptide drugs can now be produced in a straightforward way through synthetic methods and many techniques have been developed for improved peptide stability.

Therefore, the idea of transferring the protein properties to smaller systems whilst conserving the DNA recognition ability has received considerable attention. The bZIP leucine zipper TF binds double-stranded DNA as a dimer, presented as uninterrupted α-helices that grip the major groove and interact with the DNA through basic residues. The main residues involved in DNA recognition are the amino acids 226-248, located at the N-terminal basic region of the GCN4 protein[8]. The optimal position and spatial arrangement of the dimeric peptides is ensured by the leucine zipper domain. Simplified models of these proteins have been designed based on the substitution of the dimerization domain by a scaffold for the appendage of the basic region peptides in a correct geometry[9]. First proof of this concept was delivered by Kim et al.[10] using a disulfide bond as connector between the extended basic region of the peptides. Since then, several models have been developed trying to improve upon the pioneer one[11–15].

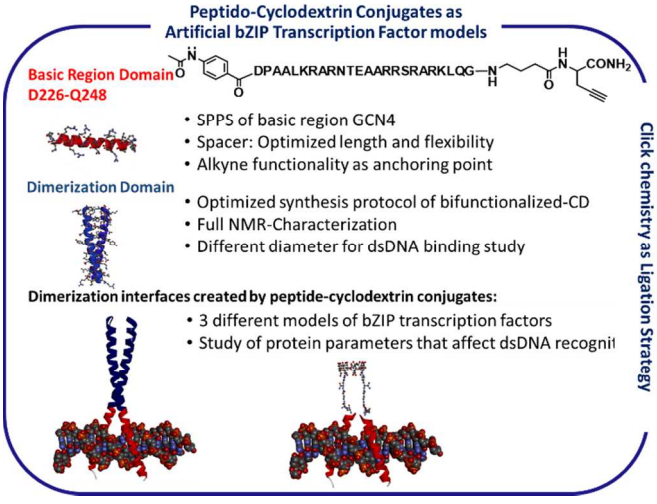

Figure 1: Design of cyclodextrin based DNA binders.

Herein we report on cyclodextrin based TF models where the dimerization domain of the GCN4 protein is substituted by a scaffold which allows accurate regulation of the geometry and the

mutual distance between the two peptide strands. This has been achieved by the use of α, β and γ-cyclodextrin derivatives in order to scan and evaluate the importance of the distance between the two anchoring points for the dimerizing peptides (Figure 1). Due to the rigid structure of the cyclodextrins, attachment of the basic region peptide of the GCN4 protein onto opposite positions of the primary rim of the cyclodextrins indeed allows control of the dimerization distance. Based on the diameters of the primary rim of α, β and γ–cyclodextrins, which are 5.7, 7.8 and 9.5 Å respectively[16], three different peptide-cyclodextrin conjugates have been synthesized, keeping in mind that the width of the major groove is 11.2 Å. For this purpose, we have synthesized $6^{I},6^{IV}$-diazido-α-cyclodextrin, $6^{I},6^{IV}$-diazido-β-cyclodextrin and $6^{I},6^{V}$ - diazido-γ-cyclodextrin, (Figure 2), in order to conjugate peptides which are functionalized with an alkyne at the C-terminus via CuAAC.

The CuAAC is a broadly developed strategy for the bioconjugation of peptides to different building blocks due to its mildness, compatibility with a large variety of functional groups and the possibility of performing the reaction in wide range of solvents and buffers. In view of the specific geometry, steric and electronic properties, a 1, 2, 3-triazole can be regarded as a trans-amide bond mimic[17]. Moreover, this linkage is stable under physiological conditions, thus representing a perfect heterocyclic moiety to replace unstable linkers[18–20]. In addition, successful replacement of dipeptide sequences in α-helical peptides by triazole units has been shown to only insignificantly influence the secondary peptide structure[17].

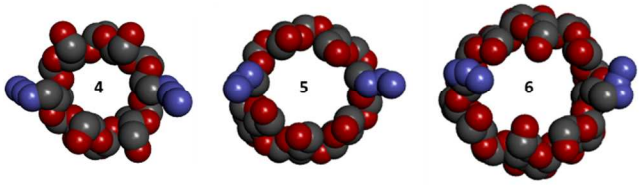

Figure 2. Molecular visualization of the cyclodextrin derivatives synthesized as dimerization domains. From left to right: $6^{I},6^{IV}$-diazido-α-cyclodextrin **4**, $6^{I},6^{IV}$-diazido-β-cyclodextrin **5** and $6^{I},6^{V}$-diazido-γ-cyclodextrin **6**. Exact structures are described in SI.

Peptide-cyclodextrin conjugates have been previously developed for a broad range of applications, such as drug release systems[21,22], enantioselective ester hydrolysis catalysis[23], and for the creation of new types of chemosensors[24], enzyme mimics[25] and self-assembling materials[26,27]. Although CD conjugation to peptides has been intensively studied, we here for the first time use the cyclodextrin moiety as artificial dimerizing unit for mimicking protein-DNA interactions.

Results and discussion

The required diazido cyclodextrin derivatives **1-3**,were prepared by conversion of the known $6^{I},6^{IV}$-dibromo-α-cyclodextrin, $6^{I},6^{IV}$-dibromo-β-cyclodextrin[28] and $6^{I},6^{V}$-dibromo-γ-cyclodextrin[29] to compounds **4-6** (Figure 3) in one step by reaction with three-fold excess of sodium azide in DMF at 50°C. Pure compounds **4-6** were isolated from the reaction mixtures by reversed-phase column chromatography with yields of 90%, 87% and 77% for the α, β and γ-cyclodextrin derivatives, respectively.

As we have previously illustrated, peptides attached directly to a dimerization unit are unable to adopt an adequate position for major groove binding[30]. Thus, a spacer is needed between the dimerization scaffold and the basic region GCN4 peptide to afford some flexibility required for binding. In addition to that, a C-terminal propargylglycine residue was introduced for conjugation of the deprotected peptide to the azide containing cyclodextrin scaffolds. The GCN4 basic region consists of the following 23 amino acids: DPAALKRARNTEAARRSRARKLQ which specifically recognize the ATF/CREB-binding site (5'-ATGA C/G TCAT-3'). The monomeric GCN4 sequence was synthesized using standard Fmoc SPPS on a Rink-amide ChemMatrix resin. After cleavage and deprotection, the peptide could be used for the next steps without intermediate purification.

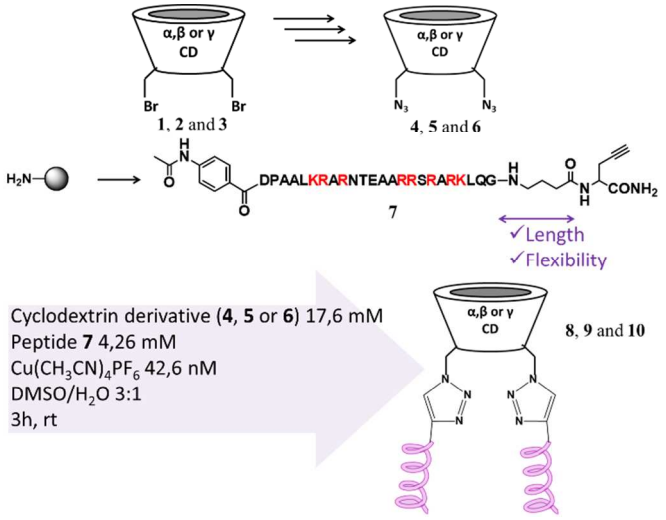

Figure 3. Synthesis scheme for the conjugation of the different cyclodextrins **4**, **5** and **6** and peptide **7** to obtain final compounds **8**, **9** and **10**. Residues that can chelate copper are marked in red.

The basic region GCN4 peptide functionalized with an alkyne at the C-terminus was then anchored to opposite positions at the primary rim of α, β and γ-cyclodextrin derivatives (Figure 3). In order to prevent aggregation of the peptide during the reaction, high polarity media were needed. In addition, polar solvents prevent copper species from aggregation. Therefore, we chose DMSO/H_2O as a solvent mixture. It is known that sodium ascorbate can cause covalent modifications of lysines and arginines, and can form reactive oxygen species[31,32]. Therefore, $Cu(CH_3CN)_4PF_6$ was chosen as catalyst after carefully studying different reaction conditions. It is to be noted that a high excess of catalyst is needed which can probably be ascribed to the chelation of copper by the nitrogen containing amino acid side chains, which are intensely represented within this particular DNA binding peptide sequence. The reaction was stopped after 3 hours at room temperature under argon. Final purification of the reaction mixture was achieved via RP-HPLC and all conjugates could be obtained in high purity (Figure 4).

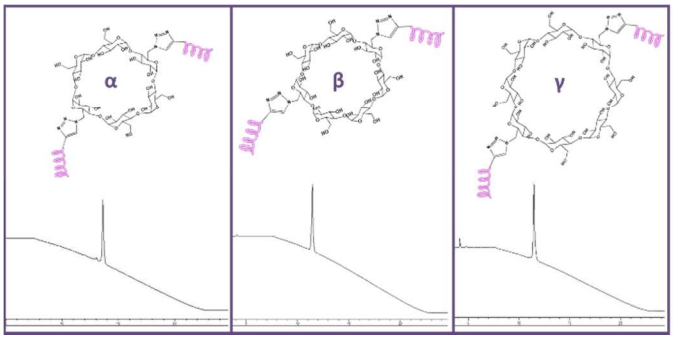

Figure 4. Structure of final compounds **8** (α), **9** (β) and **10** (γ) and RP-HPLC chromatograms of purified compounds (0-100% ACN in 15 min on C4, 300 Å).

After successful synthesis of the constructs, the DNA binding capabilities of the systems were investigated. Quantitative binding analysis was performed by Electrophoretic Mobility Shift Assay (EMSA) using double stranded ^{32}P-labeled CRE DNA (CRE: 5'-ATGACGTCAT-3'), which is the natural palindromic binding site of GCN4 protein. This technique allows the sensitive determination of the dissociation constant (Kd) (Figure 5).

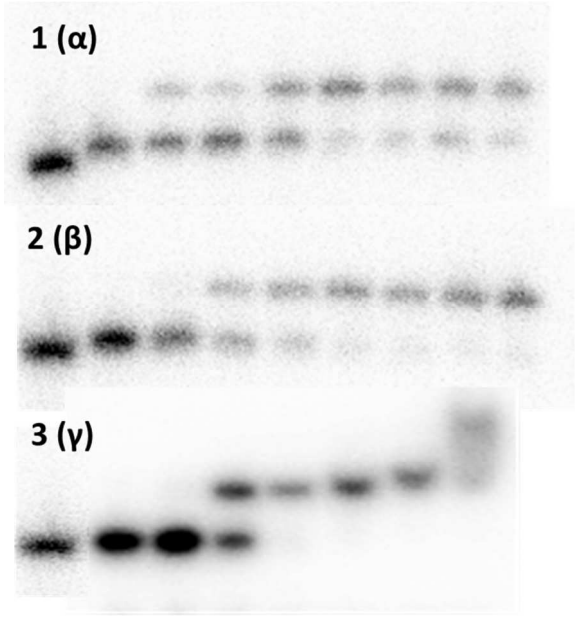

Figure 5. EMSA titration at 4°C of the dipodal peptidocyclodextrin conjugates to the 5'-labeled ^{32}P-CRE sequence (CRE: 5'-CGGATGACGTCATTTTTTTTC-3' underlined portion indicates the binding region): Concentrations: 5 nM dsDNA; First lane in all the gels: pyrimidine strand. Concentration of peptide in the lanes 2-9 for gel 1 and 2 (8 and 9): 0, 0.05, 0.0625, 0.075, 0.0875, 0.1, 0.1125 and 0.125 µM. Concentration of peptide in the lanes 2-8 for gel 3 (10): 0, 0.06, 0.125, 0.25, 0.5, 1, 2 µM. A Fuji FLA3000 phosphorimager was used for gel analysis and Multi Gauge V 3.0 software (Fujifilm) for quantification of the electrophoretic band intensities.

The results show all conjugates to bind to the target DNA sequence as evident from the appearance of an up-shifted band. In contrast no binding was observed when incubating the conjugates with random DNA sequences (see SI). Previous models developed by Morii and Mascareñas possess the same length of the basic region peptides (D226-Q248) and are therefore comparable with our systems. The group of Morii developed a system in which the dimerization unit consisted of a complex of cyclodextrin and adamantane. Both counterparts were anchored to a GCN4 basic region peptide and bind only when dimerized. EMSA studies were performed with < 1 nM radiolabelled CRE, obtaining a Kd < 150 nM[11]. More recent models designed by the group of Mascareñas employed a diazobenzene moiety for dimerization, with a Kd < 5 nM using < 1 nM radiolabelled CRE. They also reported the affinity of a disulfide-bridged GCN4 basic region, with a Kd of < 150 nM approximately for a DNA concentration of 50 nM[33]. The latest model represents a dual mimic in which dimerization arrangement and DNA selectivity are controlled by selected external parameters[34]. The basic region peptides were functionalized with a cysteine and a terpyridine moiety attached to the residues at the N- and C- termini of the peptide chain to achieve selective dimerization at both sites. Depending on the conditions, the disulfide-based or the metal-terpyridine complex-based dimers were preferred, and different DNA sequences could be targeted. By dimerizing the peptide in the presence of Ni^{2+}, the construct bound to the CRE sequence with a Kd of 299±26 nM, using a DNA concentration of 100 nM, ~100 pM labeled with ^{32}P. For these 3 last models, it should be noted that the nonionic detergent (NP-40) and BSA were used during EMSA, known to decrease aggregation of peptides and

proteins and therefore favor the interaction with the DNA[35,36]. In our case, the obtained Kd values at 4°C for compounds **8**, **9** and **10** were 50 ± 20, 30 ± 20 and 100 ± 60 nM respectively. A rough comparison thus indicates that our new constructs, apart from being synthesized in a straightforward and convergent manner, display comparable binding properties to those described in literature. The obtained values are further within the same order of magnitude as those calculated for the binding of bacterially expressed GCN4 and synthetic versions thereof. For instance, a dimer comprising of a 56mer GCN4 basic region (residues 226-281) also binds CRE in the nanomolar range (Kd ~12 nM)[37–39]. No data for the dissociation constant of the natural protein have been reported so far.

In order to better explain the results obtained from the binding pattern on the gels and the determination of the Kd values, we performed a molecular visualization of the dimerization interface of the bZIP GCN4 TF obtained by discarding the leucine zipper domain from the crystal structure of the natural protein (Figure 6, D). The distance between the C-termini of the basic regions is shown in figure 6 at different perspectives of the protein-DNA interface, and is found to be 15.052 Å. The Kd values showed that derivative **9** was the one with the best binding capability to the CRE sequence. Therefore, we consider that beta CD is the dimerizer which allows optimal anchoring of the peptides on the major groove of the DNA. In the protein the C-termini are placed at a distance of approximately 15 Å. In case of our constructs, although the diameter of the primary rim of the cyclodextrins is known (Figure 6, left), due to the flexibility of the linker, the exact distance between the C-termini of the peptides on **8**, **9** and **10** cannot be predicted accurately. However, we observed that increasing or decreasing the distance between the attachment points of the peptides by the use of different CD scaffolds results in a deviation of the optimum geometry of the system. This is reflected in terms of decreased binding affinity and lower Kds. It should also be noted that these results are specific for the basic region GCN4 peptide sequence and the given spacers.

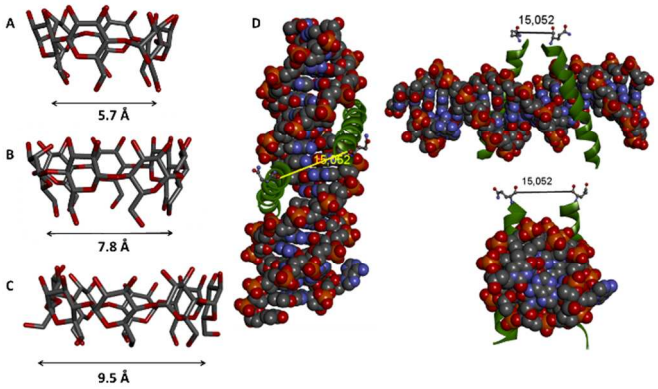

Figure 6. 3D structure of α, β and γ-cyclodextrin and primary rim diameter distance (A, B and C respectively) and crystal structure of the GCN4 basic region peptides appended to the major groove of the DNA from different perspectives. Distance between C-termini indicated in angstroms (PDB: 1YSA) (D).

Conclusions

In conclusion, three peptide-cyclodextrin conjugates for sequence-selective DNA recognition were obtained. This was achieved by the use of α, β and γ-cyclodextrin diazido derivatives as scaffolds for the appendage of the peptides by CuAAC. We have successfully synthesized and fully characterized $6^{I},6^{IV}$-diazido-α-cyclodextrin (**4**), $6^{I},6^{IV}$-diazido-β-cyclodextrin (**5**) and 6^{I}, 6^{V}-diazido-γ-cyclodextrin (**6**). Though examples exist of CuAAC reactions with CD derivatives[26,40], to the best of our knowledge long, deprotected peptides of this size have so far not been conjugated to cyclodextrins via CuAAC. We here present optimized conditions for the anchoring of such long peptides to cyclodextrin units via click chemistry.

Our results indicate the usefulness of an optimized dimerization configuration between both peptides in artificial TF models. Indeed, it was shown that the distance between the anchoring points has a notable influence on DNA binding. Successful models can be obtained by trying to approach the exact features of the protein at the interface between the dimerization and the basic region domains of the bZIP TF to achieve DNA binding comparable to that of the natural TF.

Experimental

Materials

All organic solvents were purchased from commercial suppliers and used without further purification or drying. DMF extra dry (with molecular sieves, water < 50 ppm) was acquired from ACROS Organics. DMF an NMP (peptide synthesis grade) were purchased from Biosolve. Ethyl Acetate, Acetonitrile, Methanol, Diethyl Ether, DIPEA, supplied as extra dry, redistilled, 99.5 % pure and Triisopropylsilane were purchased from Sigma Aldrich. Water with the Milli-Q grade standard was obtained in-house either from a Millipore ROs 5 purification system or a Sartorius Arium 611 DI. Rink-Amide ChemMatrix (100-200 μm, manufacturer's loading: 0.52-0.54 mmol/g) was obtained from Biotage. All reagents were acquired from commercial sources and used without prior purification. Fmoc-Propargylglycine-OH (Fmoc-Pra-OH), tris-borate-EDTA buffer 10x pH 8.3 (TBE buffer), ammonium persulfate (APS), tetramethylethylenediamine (TEMED), PyBOP and HBTU coupling reagents were obtained from either Merck Novabiochem or IRIS Biotech GmbH, while HATU (purum ≥ 98.0 %) was acquired from Fluka. TFA was obtained from IRIS Biotech GmbH. The Nα-Fmoc protected amino acids were purchased at Merck Novabiochem, IRIS Biotech GmbH and Fluka, or supplied by MultiSynTech GmbH. All chiral α-amino acids possessed the L configuration. Throughout this work, residues with standard acid-sensitive side-chain PGs were used: Asp(OtBu) [D], Cys(Trt) [C], Glu(OtBu) [E], His(Trt) [H], Lys(Boc) [K], Asn(Trt) [N], Gln(Trt) [Q], Arg(Pbf) [R], Ser(tBu) [S], Thr(tBu) [T]. All oligonucleotides used were commercially purchased from Eurogentec (HPLC purified using RP-cartridge-Gold, 200 nm scale) and were used as such.

Peptide syntheses

All automated peptide syntheses were synthesized on Rink Amide Chemmatrix resin with a loading of 0.54 mmol/g using 10 eq. of Amino Acid, 10 eq. HBTU and 20 eq. DIPEA using Fmoc/tBu SPPS. The coupling time was 1 h. Attachment of the first two

residues Glycine and GABA were performed manually. This was done to increase the space between the scaffold and the peptide. Residues D226-Q248 were then coupled via automated peptide synthesis. 20% piperidine/NMP was used for Fmoc deprotection. As a last residue and to increase UV-absorption and facilitate HPLC analysis, 4-acetamidobenzoic acid was coupled to the N-terminus. Cleavage and deprotection of the peptide was performed with a cocktail mixture containing TFA/TIS/H_2O (95/2.5/2.5) for 4 hours at room temperature. The peptide was obtained by precipitation with cold ether and lyophilization. No further purification was performed at this stage.

Peptide-CD conjugation via CuAAC

The selected deriviatized Cyclodextrin scaffold **4**, **5** or **6** (4 eq.) was dissolved in dry DMSO in a round bottom flask. Peptide **7** (1 eq.) was dissolved in milliQ water and added it to the reactor. $Cu(CH_3CN)_4PF_6$ (10 eq.) was dissolved in dry DMSO and added to the reaction mixture. The reaction was stirred for 3h at room temperature under argon. The reaction was monitored by RP-HPLC and purified by fraction collection in RP-HPLC to obtain compound **8**. Fractions were lyophilized and analyzed by RP-HPLC and MALDI-TOF.

Electrophoretic mobility shift assay (EMSA) for quantification of the dissociation constant

Preparation of ^{32}P-labeled double-stranded DNA target: Oligonucleotide CRE (5' – CGG ATG ACG TCA TTT TTT TTC – 3') was 5'-labeled using [γ-^{32}P] ATP and T4 polynucleotide kinase (Fermentas) according to the manufacturer's protocol and then purified using QIAquick Nucleotide Removal Kit (Qiagen). The 5'-end labeled pyrimidine oligonucleotide was annealed with the unlabeled complementary strand. An amount of 5nM dsDNA was prepared diluting 20 µL 0.5 M Tris, pH = 8, 40 µL 2.5 M NaCl, 40 µL 0.025 M EDTA and then adding milliQ water such that the total volume is 1 mL. The DNA was annealed in a heat block by heating from 95°C during 5 minutes and then slow cooling to room temperature. Loading buffer: 20 µL Tris 1 M, pH = 7.6, 20 µL KCl 0.2 M, 20 µL $MgCl_2$ 0.1 M, 40 µL EDTA 0.025 M. Sucrose: 30% sucrose in mQ (300 mg/mL) Peptides: 10 µL stock solutions (10x) were prepared in MiliQ water. First set of experiments were performed with 10x solutions of **8**, **9** and **10** at concentrations of 0, 0.6, 1.25, 2.5, 5, 10 and 20 µM. Loading mixture: The loading mixture comprised of: 10 µL mQ, 4 µL sucrose, 2 µL loading buffer, 2 µL DNA, 2 µL peptide. The loading mixture was prepared only 1 hour prior to running of gels and kept on ice as soon as ready.
Preparation of Gels (for 1 Gel): In a clean In a glass beaker the following were added (in given order): 21.57 mL mQ, 0.6 mL 10x TBE, 7.5 mL of 40% acrylamide solution (29:1), 0.3 mL APS (10% w/w in mQ) and 30 µL of TEMED was then added to the mixture and mixed properly before pouring it gently along parallel glass plates. The glass plates were tapped gently to ensure removal of all air bubbles and the markers were squeezed between the plates to ensure uniform width of each well. Sufficient time was given for polymerization (40 minutes).
Electrophoresis: A pre-run of the gels was performed prior to loading them. Care was taken to see that the gels were properly immersed in 0.2x TBE buffer (non-denaturing gel, without urea) and the loading wells were free from any air bubbles. The wells were washed after the pre-run. Instrument settings: 150 V, 100 mA, 19 W for 30 minutes with circulation water-cooling. 5 µL of the loading mixture was then loaded onto the wells. Instrument settings: 150 V, 100 mA, 19 W for 45 minutes with circulation water-cooling.
Analysis of gels: The gels were frozen and analyzed by phosphor imaging using Molecular Imager FX and the data were processed using Quantity One software (BioRad).

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Notes and references

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† Electronic Supplementary Information (ESI) available: Reaction conditions, RP-HPLC traces, ESI-MS, NMR spectra and EMSA studies.. See DOI: 10.1039/b000000x/

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