Towards identifying preferred interaction partners of fluorinated amino acids within the hydrophobic environment of a dimeric coiled coil peptide†

Toni Vagt,‡ Elisabeth Nyakatura, Mario Salwiczek, Christian Jäckel§ and Beate Koksch*

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Phage display technology has been applied to screen for preferred interaction partners of fluoroalkyl-substituted amino acids from the pool of the 20 canonical amino acids. A parallel, heterodimeric α-helical coiled coil was designed such that one peptide strand contained one of three different fluorinated amino acids within the hydrophobic core. The direct interaction partners within the second strand of the dimer were randomized and coiled coil pairing selectivity was used as a parameter to screen for the best binding partners within the peptide library. It was found that despite their different structures, polarities and fluorine contents, the three non-natural amino acids used in this study prefer the same interaction partners as the canonical, hydrophobic amino acids. The same technology can be used to study any kind of non-canonical amino acids. The emerging results will provide the basis not only for a profound understanding of the properties of these building blocks, but also for the *de novo* design of proteins with superior properties and new functions.

Introduction

The introduction of fluorine has proven to be a successful concept for improving the biological and pharmaceutical properties of drug candidates.[1] The unique properties of fluorine as well as its absence from the pool of canonical amino acids make fluorinated amino acids a promising tool in the development of peptide-based drugs.[2–4] However, the application of fluorinated amino acids for rational protein design depends on a comprehensive knowledge of their properties within a native protein environment.[5] The systematic evaluation of these aspects requires model systems that allow the sensitive detection of the influence of fluorinated amino acids on peptide conformation and stability. A protein-folding motif, which suitably conforms to these requirements, is the α-helical coiled coil.[6,7] The molecular basis for structure formation of this folding motif has been extensively studied and our current understanding of its design principles enables the design of peptide models with well defined properties.[8]

Typically, a coiled coil consists of two to five right-handed α-helices wrapped around each other in a left-handed twist. The primary structure is formed by a periodical repeat of seven amino acids denoted $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$. Positions **a** and **d** are commonly occupied by hydrophobic residues such as leucine, isoleucine and valine, which form a hydrophobic surface and provide the driving force for oligomerization under aqueous conditions. Normally, charged amino acids are placed at positions **e** and **g**. These form a second interaction domain and further contribute to coiled coil stability and folding specificity through the formation of inter-helical salt bridges. The remaining positions are occupied by predominantly hydrophilic amino acids and are not involved in the inter-helical interactions of dimeric coiled coils.[8,9]

Several studies have shown that extensive substitution of the apolar residues at positions **a** and **d** by highly fluorinated amino acids results in stabilization of the coiled coil, which suggests a strong favourable interaction between the fluoroalkyl groups.[10–13] On the other hand, single amino acid substitutions may have a destabilizing effect on the stability of coiled coil peptides.[14,15] However, aside from these effects on the structural stability of peptides and proteins, less is known about the preferred interacting partners of fluorinated amino acids in a natural polypeptide environment.

As an understanding of these properties is required for the efficient application of fluorinated amino acids in drug design, a screening system to determine the interaction potential of these building blocks is of general interest. Using a heterodimeric coiled coil model system, we were able to investigate the influence of single fluorinated amino acids on the stability of this folding motif and obtained useful information about the impact of their steric demand and hydrophobicity.[16] Based on the same peptide model system, we recently described a phage display[17,18]-based screening strategy that allows the determination of preferred interactions within the hydrophobic core.[19] Here, we describe the use of this screening system to investigate the interaction possibilities of fluorinated amino acids within the hydrophobic environment of the coiled coil interaction domain. The predominant goal of this study was to determine the best binding partners for coiled coil strands bearing fluorinated amino acids.

Results and discussion

Our starting point was the *de novo* designed heteromeric coiled coil pair VPE/VPK, which forms parallel dimers.[16] Several

Institute of Chemistry and Biochemistry–Organic Chemistry, Freie Universität Berlin, Takustraße 3, 14195, Berlin, Germany. E-mail: koksch@chemie.fu-berlin.de

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‡ Present address: Institute of Molecular Biology and Biophysics, ETH Zürich, CH-8093 Zürich, Switzerland

§ Present address: Laboratory of Organic Chemistry, ETH Zürich, CH-8093 Zürich, Switzerland

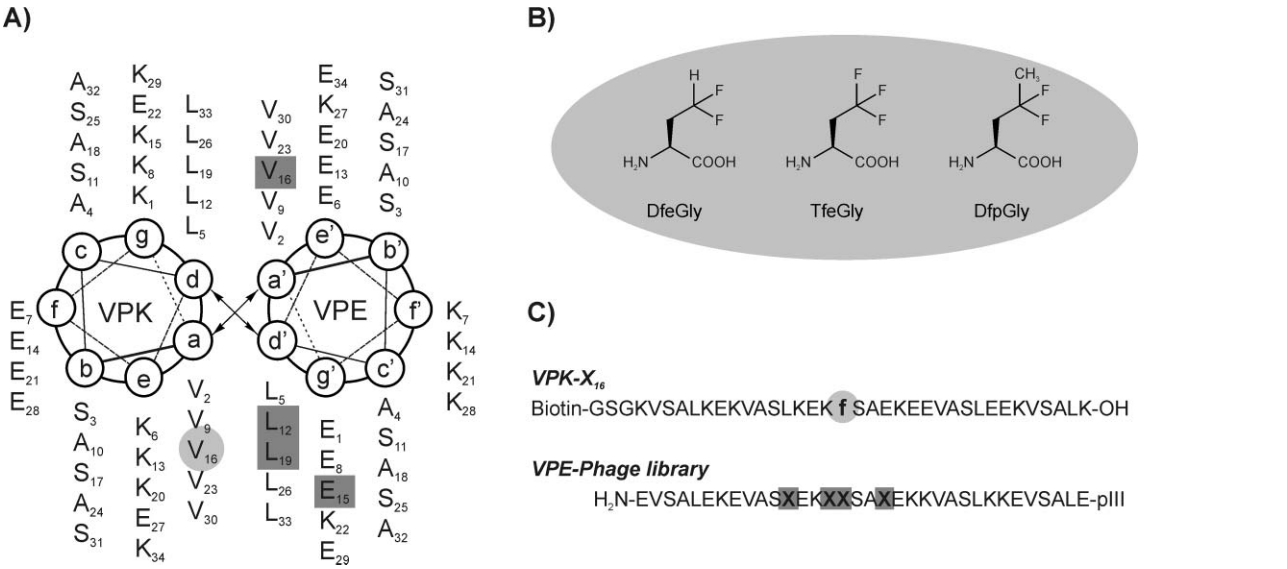

Fig. 1 (A) Helical wheel presentation of the parallel VPK/VPE heterodimer. The substitution position $\mathbf{a}_{16}$ in VPK is labeled by a grey circle, the four randomized positions in VPE are highlighted by dark grey squares. (B) Structure of the three fluorinated amino acids incorporated into position $\mathbf{a}_{16}$ of VPK. (C) Amino acid sequence of VPK and the VPE library. **X** indicates each of the 20 canonical amino acids, while **f** stands for the fluorinated amino acids (*S*)-2-amino-4,4-difluorobutanoic acid (DfeGly), (*S*)-2-amino-4,4,4-trifluorobutanoic acid (TfeGly) and (*S*)-2-amino-4,4-difluoropentanoic acid (DfpGly), respectively.

fluoroalkyl-substituted amino acids were incorporated in position $\mathbf{a}_{16}$ of VPK as part of the hydrophobic core. Because the hydrophobic positions **a** and **d** provide the driving force for recognition of both peptide strands and a surface of strong side-chain–side-chain interactions, this region allows investigation of the interaction mode of artificial residues at these sites.[14,15] Consequently, the direct interaction partners of position $\mathbf{a}_{16}$ in the interacting VPE strand, which are $\mathbf{d'}_{12}$, $\mathbf{g'}_{15}$, $\mathbf{a'}_{16}$ and $\mathbf{d'}_{19}$, were randomized (Fig. 1).

The resulting VPE-library was displayed on the surface of bacteriophage M13 fused to its minor coat protein pIII and coiled coil pairing selectivity was used to select the best binding partners for several substituted VPK peptides out of the pool of VPE-variants.

According to our previous work, (*S*)-2-amino-4,4-difluorobutanoic acid (DfeGly), (*S*)-2-amino-4,4,4-trifluorobutanoic acid (TfeGly) and (*S*)-2-amino-4,4-difluoropentanoic acid (DfpGly) were chosen as substitutes for Val_{16} in VPK (Table 1).

It was shown that the incorporation of DfeGly, TfeGly and DfpGly into two different coiled coil systems, and at different positions within the hydrophobic core, generally has a destabilizing effect compared to the amino acids valine and leucine, which naturally occur at these positions.[8] However, it was also shown that the impact of the fluoro-substitution also depends on subtle structural differences of the protein environment itself. In view of the destabilizing effects we observed for position $\mathbf{a}_{16}$ of VPK, we expected to find VPE mutants possessing better and more specific binding partners for the fluoroalkyl-substituted VPK-variants. Our expectations notwithstanding, the VPE-variants selected by panning using different fluorinated VPK-peptides as targets were quite similar to the sequences that were selected for the wild type (Val), in which hydrophobic amino acids strongly dominate in all positions (Table 2).

The amino acids selected for the variable positions in principle follow the pattern Leu(12)Leu(15)Ile(16)Tyr(19) (VPE-$L_{15}I_{16}Y_{19}$) or Leu(12)Tyr(15)Ile(16)Leu(19) (VPE-$Y_{15}I_{16}$). Leucine already occurred in the **d′**-positions of VPE wild type and its selection in this position is in agreement with the general preference of position **d** for this amino acid in parallel coiled coil dimers. The selection of isoleucine in position $\mathbf{a'}_{16}$ can be explained by the fact that hydrophobic, β-branched amino acids are the most stabilizing amino acids in **a**-positions of parallel coiled coil peptides.[8] However, the selection of tyrosine in $\mathbf{g'}_{15}$ and $\mathbf{d'}_{19}$ was somewhat unexpected. Possibly, cation–π interactions between tyrosine and lysine in the opposite **g′**-positions of VPK favour

Table 1 Peptide sequences of the VPK-variants used for the phage display screening

Peptide	Sequence
Bio-VPK	Biotin-GSGKVSALKEKVASLKEK ***V*** SALKEEVASLEEKVSALK-OH
Bio-VPK-N_{16}	Biotin-GSGKVSALKEKVASLKEK ***N*** SALKEEVASLEEKVSALK-OH
Bio-VPK-$DfeGly_{16}$	Biotin-GSGKVSALKEKVASLKEK-***DfeGly***SALKEEVASLEEKVSALK-OH
Bio-VPK-$DfpGly_{16}$	Biotin-GSGKVSALKEKVASLKEK-***DfpGly***SALKEEVASLEEKVSALK-OH
Bio-VPK-$TfeGly_{16}$	Biotin-GSGKVSALKEKVASLKEK-***TfeGly***SALKEEVASLEEKVSALK-OH

Table 2 Amino acids selected for the randomized positions in VPE after 5 rounds of panning against the various VPK-peptides

	Position $\mathbf{d'}_{12}$	**Position $\mathbf{g'}_{15}$**	**Position $\mathbf{a'}_{16}$**	**Position $\mathbf{d'}_{19}$**
VPK	Leu	Tyr or Leu	Ile	Leu or Tyr
VPK-$DfeGly_{16}$	Leu	Hydrophobic amino acids	Ile	Tyr or Leu
VPK-$DfpGly_{16}$	Leu	Leu or Tyr	Ile	Leu or Tyr
VPK-$TfeGly_{16}$	Leu or Phe	Leu or Tyr	Ile	Tyr or Leu

selection of this amino acid.[20] The occurrence of tyrosine in position $\mathbf{g'_{15}}$ seems to be more favourable because of the stronger steric hindrance of the aromatic side chain in position $\mathbf{d'_{19}}$. As exclusively hydrophobic amino acids were selected in position $\mathbf{g'_{15}}$, a VPE variant bearing leucine in both $\mathbf{d'}$ positions as well as in position $\mathbf{g'_{15}}$ (VPE-$L_{15}I_{16}$) is also expected to form a favourable binding partner for the VPK wild type and for the fluorinated VPK variants.

Apolar amino acids were selected in position $\mathbf{g'_{15}}$ of VPE when hydrophobic amino acids were placed in position $\mathbf{a_{16}}$ of the target peptide. This results in an extension of the hydrophobic core, which presumably contributes to coiled coil stability. In contrast, panning with a VPK-variant that bears asparagine in position $\mathbf{a_{16}}$ instead of valine (VPK-Asn_{16}) as the target resulted in selection of a different VPE-peptide. Here, probably caused by the different steric demand of asparagine, leucine was preferably selected in position $\mathbf{a'_{16}}$ and glutamic acid occurred in position $\mathbf{g'_{15}}$ (T_m of the phage display selected VPK-Asn_{16}/VPE-Leu_{16} couple is 45.5 °C, T_m of VPK-Asn_{16}/VPE wild type is 33.4 °C). Here, the polar asparagine in position $\mathbf{a_{16}}$ of VPK cannot contribute to an extension of the hydrophobic core. Consequently, glutamic acid was selected in position $\mathbf{g'_{15}}$ of VPE, which favours coiled coil formation by inter helical electrostatic interactions. These results confirm the potential of the described screening method to react on specific properties of varying amino acid side chains in the target peptide by the selection of different VPE variants.

To characterize the VPE variants gained as binding partners for the fluorinated VPK variants in detail, these VPE peptides (VPE-$L_{15}I_{16}Y_{19}$, VPE-$Y_{15}I_{16}$, VPE-$L_{15}I_{16}$) were chemically synthesized and the thermal stability of all coiled coil couples, which arose from the combination with the different VPK-variants, was determined (Fig. 2). Compared to the original VPK-variant/VPE couple, a distinct increase of thermal stability was observed for the phage display selected VPE-peptides with each of the VPK-variants. Irrespective of the differing frequency with which the VPE variants were selected for the modified VPK peptides, each fluoroalkyl-substituted VPK peptide, as well as the VPK wild type, was found to form the most stable coiled coil in combination with VPE-$L_{15}I_{16}$ (Table 3 and 4). Accordingly, the investigated non-natural amino acids DfeGly, DfpGly and TfeGly prefer the same amino acid pattern within the corresponding coiled coil strand. Moreover, the trend in stability is basically the same between the different VPK-variants, irrespective of the corresponding VPE-peptide. In each case, VPK wild type forms the most stable coiled coil assembly, while VPK-$TfeGly_{16}$ and VPK-$DfpGly_{16}$ form less stable coiled coil structures with only minor differences among each other. VPK-$DfeGly_{16}$ forms the least stable coiled coil in combination with each VPE variant, including the VPE wild type (Fig. 2). This observation is plausible since the DfeGly side chain shows both a lower hydrophobicity and smaller van der Waals volume than valine or the other fluorinated amino acids.[21]

Table 3 Peptide sequence of the VPE variants selected as interaction partner of the different VPK peptides

Peptide	Sequence
VPE-$L_{15}I_{16}$	H-EVSALEKEVASLEKLISALEKKVASLKKEVSALE-OH
VPE-$Y_{15}I_{16}$	H-EVSALEKEVASLEKYISALEKKVASLKKEVSALE-OH
VPE-$Y_{15}I_{16}L_{16}$	H-EVSALEKEVASLEKLISAYEKKVASLKKEVSALE-OH

Table 4 Thermal stability (T_m) of the different VPK peptides substituted in position $\mathbf{a_{16}}$ by DfeGly, DfpGly and TfeGly in combination with the preferred VPE variants selected by phage display

	VPE-$L_{15}I_{16}$	VPE-$Y_{15}I_{16}$	VPE-$L_{15}I_{16}Y_{19}$	VPE
VPK (Val_{16})	74.9 °C	74.0 °C	72.1 °C	72.4 °C
$TfeGly_{16}$	74.4 °C	70.3 °C	69.0 °C	69.4 °C
$DfpGly_{16}$	74.2 °C	70.9 °C	70.4 °C	70.1 °C
$DfeGly_{16}$	73.3 °C	67.5 °C	65.3 °C	63.9 °C

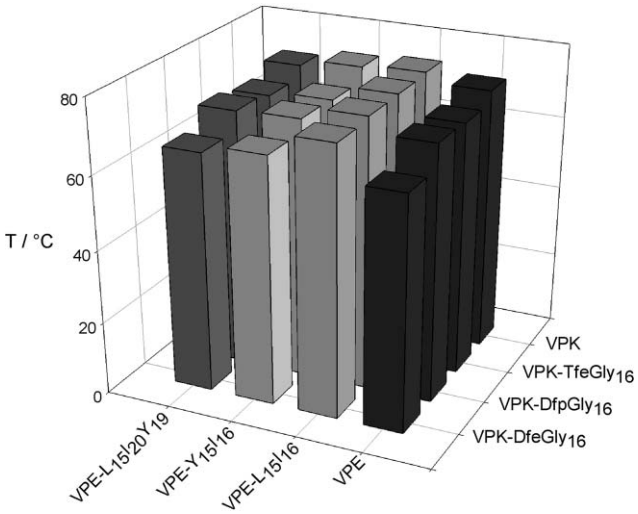

Fig. 2 Illustration of the thermal stability of coiled coil couples formed by the DfeGly, DfpGly and TfeGly substituted VPK peptides as well as the VPK wild type in combination with different VPE variants. The melting points (T_m) are listed in Table 4.

The extension of the hydrophobic interface, which is caused by the selection of predominantly hydrophobic amino acids in position $\mathbf{g'_{15}}$, supposedly compensates for the effects caused by the fluorinated amino acids. The additional hydrophobic amino acid position stabilizes the coiled coil structure, which basically relies on hydrophobic interactions and reduces the sensitivity for changes in position $\mathbf{a_{16}}$. To avoid this effect and to check if any differences in the selection results can be detected, a second library was constructed in which position $\mathbf{g'_{15}}$ remained unmodified and solely the hydrophobic core positions $\mathbf{d'_{12}}$, $\mathbf{a'_{16}}$ and $\mathbf{d'_{19}}$ were randomized.

The VPE peptides that were selected by screening this library for the VPK wild type, and the DfeGly-, DfpGly- and TfeGly-substituted analogues, show little variance and preferably follow the pattern Leu(12)Ile(16)Leu(19). Except for position $\mathbf{g'_{15}}$, this peptide sequence in principle matches those which were selected from the first library (amino acid positions $\mathbf{d'_{12}}$, $\mathbf{g'_{15}}$, $\mathbf{a'_{16}}$ and $\mathbf{d'_{19}}$ randomized). As distinguished from the extended library, leucine exclusively occurred in positions $\mathbf{d'_{12}}$ and $\mathbf{d'_{19}}$, while formerly aromatic amino acids were also selected in these positions. This observation indicates that the selection of tyrosine in hydrophobic core positions as observed for the first VPE library is a consequence of extending the hydrophobic core by position $\mathbf{g'_{15}}$ and is not specific for the investigated fluorinated amino acids.

Conclusions

In this study, we applied phage display technology to search for preferred interaction partners of fluorinated amino acids that differ in fluorine content and thus, polarity and space filling characteristics. A valine residue in a central **a**-position of a parallel coiled coil peptide was substituted by the fluorinated amino butyric acid analogues DfeGly, DfpGly or TfeGly. These peptides were used to screen phage display libraries, which were randomized at those positions of a complementary peptide that are directly interacting with the non-natural residue on coiled coil dimer formation. Interestingly, the interaction profiles of all three fluorinated amino acids were found to be similar to that of the native hydrophobic residue valine. These results complement our previous studies that investigated the effect fluorinated amino acids have on a coiled coil's structural stability. In these earlier studies, we found significant differences in impact on folding stability between the fluorinated building blocks DfeGly, DfpGly and TfeGly based on differences in steric demand and hydrophobicity.[14,15] The screening of phage libraries, however, resulted in a selection of similar interaction profiles for the fluorinated amino acids. Despite the fact that their polarity differs substantially, DfeGly, DfpGly and TfeGly, in principal, prefer a hydrophobic environment within protein structures equal to that of leucine or valine.

These results indicate that the structural diversity of the canonical amino acid alphabet is not sufficient enough to react on the differences in physicochemical properties between the investigated fluorinated side chains. That is, the spectrum of functional groups and hydrophobic structures found within the set of proteinogenic amino acid side chains fails to distinguish between subtle differences of fluoroalkyl groups. Phage display selection is based on the most stable rather than on the most specific peptide interaction. In this context, one might argue that it is the coiled coil environment of the applied system rather than the single substitution itself that determines the overall outcome of this experiment. Since substitutions by fluorinated amino acids were carried out in the hydrophobic core of a coiled coil peptide, highly hydrophobic canonical amino acids are selected for the randomized positions to stabilize the hydrophobic core. These results verify the usage of hydrophobic fluorinated amino acids when targeting naturally occurring hydrophobic interactions. In combination with additional investigations of general physicochemical and biological properties of fluorinated amino acids, such as their impact on protease stability or membrane permeability, we will gain a profound understanding of their effects in a natural protein environment. Potential applications include the design of highly selective receptor ligands and peptide-based drugs of low toxicity.

The phage display-based screening system presented in this study should be applicable to study further non-natural amino acids within a native protein environment.

Experimental

Peptide synthesis, purification and characterization

Peptide synthesis was performed on a Syro-XP-1 peptide synthesizer (MultiSynTech GmbH, Witten, Germany) using standard Fmoc/tBu chemistry as described previously.[16] Fluorinated amino acids (*S*)-2-amino-4,4-difluorobutanoic acid,[22] (*S*)-2-amino-4,4,4-trifluorobutanoic acid[23] and (*S*)-2-amino-4,4-difluoropentanoic[24] acid were synthesized as described in the literature. N-terminal coupling of biotin (Acros) was performed as double coupling (each 45–60 min) using DIC–HOBt (10 min preactivation) as coupling reagents and a fourfold excess of biotin–DIC–HOBt (1 : 1 : 1) relative to the resin loading in NMP. After synthesis, peptides were cleaved from the resin with TFA–TIS–EDT–H_2O (94 : 2.5 : 2.5 : 1) and purified by reversed phase HPLC (Phenomenex® Luna C_8, 10 µm, 250 nm × 21.2 mm). Purity and identity of the products was determined by analytical HPLC (Phenomenex® Luna C_8, 5 µm, 250 nm × 4.6 mm) and high resolution mass spectra which were recorded on an Agilent 6210 ESI-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) (see the ESI for details†).

CD spectroscopy

CD spectra were recorded on a Jasco J-715 spectropolarimeter at 20 °C (Jasco PTC-348 WI peltier thermostat). Spectra were measured in 100 mM phosphate buffer at pH 7.4 and at 20 °C (Jasco PTC-348 WI Peltier thermostat). The overall peptide concentration was 20 µM. For melting curves, the CD signal at 222 nm was recorded applying a heating rate of 3 K min^{-1} from 20 to 95 °C. All spectra were baseline corrected and each sample was prepared three times.

Peptide concentration was estimated by measuring the absorption of *o*-aminobenzoic acid which is attached at the N-terminus of each peptide as described in the literature.[16]

Construction of the VPE-library

Library construction was performed by annealing two complementary oligonucleotides that encode for the complete VPE peptide sequence and in which the library codons are randomized applying the NNK-strategy.[25,26] The library DNA was cloned into the pComb3H[27] phagemid vector (GenBank database accession number: AF268280, Barbas laboratory, TSRI) by SfiI sites and transformed into *E.coli* K12 ER2738 (New England Biolabs #E4104S) as described previously.[19] The library size was calculated to be 2.3×10^6 for library 1 (position d_{12}, g_{15}, a_{16} and d_{23} randomized) and 1.6×10^7 for library 2 (positions d_{12}, a_{16} and d_{19} randomized) (see the ESI for details†). Production of the library phage occurred as described previously.[19]

Phage selection and amplification

For phage selection, approximately 10 nmol biotinylated target peptide was immobilized at 30 µL streptavidin-coated magnetic particles (M-280, Dynal Biotech). Particles were washed twice with 500 µL 0.1% Tween 20 in PBS. 500 µL 5% non-fat dried milk in PBS was added and the sample was incubated at RT for 45 min. After removing the milk–PBS suspension, 500 µL phage solution were added and phage–target binding was performed for 1.5 h at RT. Afterwards, the particles were washed four times with 500 µL Tween20 in PBS (PBS buffer contained 0.1% Tween 20 in round 1; 1% Tween 20 in rounds 2–5; in round 5 two washing steps with 1 M GndHCl in PBS were added) and once with 500 µL TBS. Bound phages were eluted from magnetic particles by adding 25 µL freshly prepared trypsin solution (10 mg mL^{-1} in TBS) and

incubation at RT. After 30 min the reaction was quenched with 75 µL SB medium. For reinfection the received phage suspension (100 µL) was transferred to 5 mL *E. coli* culture. After 30 min at 37 °C/200 rpm, 10 µL of the cell culture were removed for output-titering and 5 mL prewarmed SB medium containing 2.5 µL carbenicillin (100 mg mL^{-1}) were added. Cells were incubated for 1 h at 37 °C/200 rpm and then transferred to 90 mL prewarmed SB medium to which 46 µL carbenicillin (100 mg mL^{-1}) and 1 mL VCSM13 helper phage (Stratagene #200251) were added. After another 1.5 h at 37 °C/200 rpm 140 µL kanamycin (50 mg mL^{-1}) were added and phages were produced over night under the same conditions. The culture was centrifuged for 30 min at 4 °C and 3000 g, and phages were precipitated by the addition of 20 vol% PEG–NaCl [20% (w/v) PEG 8000, 2.5 M NaCl] to the supernatant. After incubation for 30 min on ice phages were centrifuged for 30 min at 4 °C and 12 000 g. Isolated phages were suspended in PBS buffer and used in the following round of panning after sterile filtration (0.22 µm).

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References

1 K. Müller, C. Faeh and F. Diederich, *Science*, 2007, **317**, 1881–1886.
2 D. O'Hagan, *Chem. Soc. Rev.*, 2008, **37**, 308–319.
3 S. Purser, P. R. Moore, S. Swallow and V. Gouverneur, *Chem. Soc. Rev.*, 2008, **37**, 320–330.
4 H. Meng, S. T. Krishnaji, M. Beinborn and K. Kumar, *J. Med. Chem.*, 2008, **51**, 7303–7307.
5 N. C. Yoder and K. Kumar, *Chem. Soc. Rev.*, 2002, **31**, 335–341.
6 C. Jäckel and B. Koksch, *Eur. J. Org. Chem.*, 2005, 4483–4503.
7 M. Hakelberg and B. Koksch, *Chim. Oggi*, 2007, **25**, 48–53.
8 D. N. Woolfson, *Adv. Protein Chem.*, 2005, **70**, 79–112.
9 J. M. Mason and K. M. Arndt, *ChemBioChem*, 2004, **5**, 170–176.
10 Y. Tang and D. A. Tirrell, *J. Am. Chem. Soc.*, 2001, **123**, 11089–11090.
11 Y. Tang, G. Ghirlanda, W. A. Petka, T. Nakajima, W. F. DeGrado and D. A. Tirrell, *Angew. Chem., Int. Ed.*, 2001, **40**, 1494–1496.
12 B. Bilgiçer, X. Xing and K. Kumar, *J. Am. Chem. Soc.*, 2001, **123**, 11815–16.
13 H. Y. Lee, K. H. Lee, H. M. Al-Hashimi and E. N. G. Marsh, *J. Am. Chem. Soc.*, 2006, **128**, 337–343.
14 C. Jäckel, W. Seufert, S. Thust and B. Koksch, *ChemBioChem*, 2004, **5**, 717–720.
15 C. Jäckel, M. Salwiczek and B. Koksch, *Angew. Chem., Int. Ed.*, 2006, **45**, 4198–4203.
16 M. Salwiczek, S. Samsonov, T. Vagt, E. Nyakatura, E. Fleige, J. Numata, H. Cölfen, M. T. Pisabarro and B. Koksch, *Chem.–Eur. J.*, 2009, **15**, 7628–7636.
17 J. R. Lai, J. D. Fisk, B. Weisblum and S. H. Gellman, *J. Am. Chem. Soc.*, 2004, **126**, 10514–10515.
18 U. B. Hagemann, J. M. Mason, K. M. Müller and K. M. Arndt, *J. Mol. Biol.*, 2008, **381**, 73–88.
19 T. Vagt, C. Jäckel, S. Samsonov, M. T. Pisabarro and B. Koksch, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 3924–3927.
20 J. P. Gallivan and D. A. Dougherty, *Proc. Natl. Acad. Sci. U. S. A.*, 1999, **96**, 9459–9464.
21 S. A. Samsonov, M. Salwiczek, G. Anders, B. Koksch and M. T. Pisabarro;, *J. Phys. Chem. B*, 2009, **113**, 16400–16408.
22 D. Winkler and K. Burger, *Synthesis*, 1996, 1419–1421.
23 T. Tsushima, K. Kawada, S. Ishihara, N. Uchida, O. Shiratori, J. Higaki and M. Hirata, *Tetrahedron*, 1988, **44**, 5375–5387.
24 S. N. Osipov, T. Lange, P. Tsouker, J. Spengler, L. Hennig, B. Koksch, S. Berger, S. M. El-Kousy and K. Burger, *Synthesis*, 2004, **11**, 1821–1829.
25 J. J. Reidhaar-Olson and R. T. Sauer, *Science*, 1988, **241**, 53–57.
26 C. F. Barbas III, D. R. Burton, J. K. Scott, G. J. Silverman, *Phage Display: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 2001, Chapter 4.
27 C. F. Barbas III and J. Wagner, *Methods*, 1995, **8**, 94–103.

