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Communication

Genetic Encoding of *para*-Pentafluorosulfanyl Phenylalanine: A Highly Hydrophobic and Strongly Electronegative Group for Stable Protein Interactions

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ABSTRACT: SF_5Phe, *para*-pentafluorosulfanyl phenylalanine, is an unnatural amino acid with extreme physicochemical properties, which is stable in physiological conditions. Here we present newly developed aminoacyl-tRNA synthetases that enable genetic encoding of SF_5Phe for site-specific incorporation into proteins in high yields. Owing to the SF_5 moiety's dichotomy of strong polarity and high hydrophobicity, the unnatural amino acid forms specific and strong interactions in proteins. The potential of SF_5Phe in protein research is illustrated by (i) increasing the binding affinity of a consensus pentapeptide motif toward the β subunit of *Escherichia coli* DNA polymerase III holoenzyme by mutation of a phenylalanine to a SF_5Phe residue, (ii) site-specifically adhering β-cyclodextrin to the surface of ubiquitin, and (iii) selective detection of $^{19}F-^{19}F$ nuclear Overhauser effects in the *Escherichia coli* peptidyl-prolyl *cis*/*trans*-isomerase B following mutation of two phenylalanine residues in the core of the protein to SF_5Phe. With increasing use of the SF_5 moiety in pharmaceutical chemistry, this general method of functionalizing proteins with SF_5 groups opens unique opportunities for structural biology and *in vivo* studies.

Genetic code expansion is a powerful tool to extend the chemical diversity of proteins.[1] Over the past decade, this method has been widely applied to modify proteins with over 100 noncanonical amino acids to study protein structure and impart new function.[1,2] Because changing the function of proteins often requires precise tuning of the physicochemical properties within active sites, genetic encoding of unnatural amino acids with xenobiotic groups is particularly appealing.[3−6]

Fluorine-containing unnatural amino acids (uAA) have successfully been genetically incorporated into proteins for ^{19}F-NMR analysis to take advantage of the high selectivity with which ^{19}F-NMR signals can be observed free of background signal in biological environments.[4−6] C−F bonds are also well-known to influence ligand binding through the specific electrostatic and lipophilic properties of the fluorine atom, which often present a considerable advantage in drug development.[7] These properties are most pronounced in the pentafluorosulfanyl (SF_5) group, which possesses approximately 40% higher Hansch hydrophobicity and 9% greater electronegativity than the trifluoromethyl group.[8,9] The high electron density, conformational rigidity, and lipophilicity of SF_5 groups significantly expand the chemical space of new drugs and biologicals, but the limited synthetic accessibility of SF_5 groups presents an obstacle for their wider application.[8] Here we report a facile way for site-specific incorporation of *p*-pentafluorosulfanyl-phenylalanine (SF_5Phe) into proteins (Figure 1). Using an efficient library screening approach based on fluorescence-activated cell sorting (FACS), we identified SF_5Phe-specific orthogonal aminoacyl-tRNA synthetase (aaRS)/$tRNA_{CUA}$ pairs, which are derivatives of the tyrosyl-tRNA synthetase (TyrRS)/^{Tyr}tRNA pair from *Methanocaldococcus jannaschii* (*Mj*). The system produces SF_5Phe-containing proteins in high yield. We demonstrate enhanced binding affinity of a pentapeptide motif toward the β-clamp subunit of *E. coli* DNA polymerase III (Pol III) following mutation of a phenylalanine residue to SF_5Phe and strong

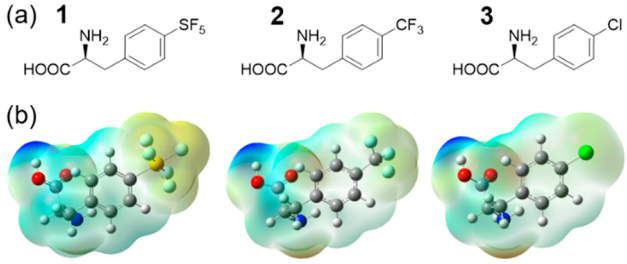

Figure 1. (a) Chemical structures of the unnatural amino acids used in this study. **1**: *p*-Pentafluorosulfanyl-phenylalanine (SF_5Phe). **2**: *p*-Trifluoromethyl-phenylalanine (CF_3Phe). **3**: *p*-Chloro-phenylalanine (ClPhe). (b) Electrostatic potential surfaces were calculated using density functional theory at B3LYP level with 6-311++G (d,p) basis set, showing SF_5Phe has a more electronegative side chain and a stronger dipole moment.

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binding of β-cyclodextrin to SF_5Phe incorporated into ubiquitin. In addition, two SF_5Phe were installed in peptidyl-prolyl *cis*/*trans*-isomerase B (PpiB), which, for the first time, allowed the selective observation of intramolecular $^{19}F-^{19}F$ nuclear Overhauser effects (NOE) in a protein.

To identify an aaRS enzyme for the incorporation of SF_5Phe into proteins in response to an amber stop codon, the library plasmid pBK-MjYRS, which sources a mutant *Mj*TyrRS and $^{Tyr}tRNA_{CUA}$, was constructed (Figure S1). The selection plasmid pBAD-GRFP encoded the amber codon interrupted fusion of mNeonGreen green fluorescent protein (GFP)[10] and mCherry red fluorescent protein (RFP)[11] (Figure S2).

Functional SF_5PheRS enzymes were screened using FACS on an Aria II high speed cell sorter. *E. coli* DH10B cells harboring both pBK-MjYRS and pBAD-GRFP were cultured under different selective conditions in subsequent screening rounds (Figures 2 and S3). Already the first positive selection round conducted in the presence of SF_5Phe (1P+) displayed more RFP-positive (R+) cells than the same experiment conducted without SF_5Phe (1P−), indicating that a large number of mutants in the tRNA synthetase library were capable of incorporating SF_5Phe. The second negative round was carried out to remove false positive *Mj*TyrRS variants that recognize endogenous amino acids. Selection was ended after the third positive round, as the ratio of R+ population between the 3P+ and 3P− samples had reached 58.1:2.4%, which indicated most of the remaining gene pool was selective as well as specific for SF_5Phe; 32 candidates that produced high RFP fluorescence were isolated and sequenced. Remarkably, all of these sequences were different (Table S2). The four SF_5PheRS enzymes that delivered the highest incorporation yields (SF20, SF61, SF87, SF100) and five randomly selected candidates (SF19, SF29, SF88, SF95, SF109) were subsequently coexpressed with a His_6-amber(TAG)-RFP reporter protein (H6RFP) encoded by the pBAD-H6RFP plasmid for mass spectrometry analysis. None of them showed mis-incorporation of endogenous amino acids (Figure S5) at the amber site. The core structures around the substrate binding pocket of SF61 (Leu32Val, Val65Ser, Met109Ala, Ala159Val, Leu162Lys) and SF100 (Leu32Val, Met109Pro, Ala159Phe, Leu162Phe) were modeled using Rosetta structure prediction tools and indicate strong hydrophobic interactions with the SF_5Phe ligand (Figure 3a,b).

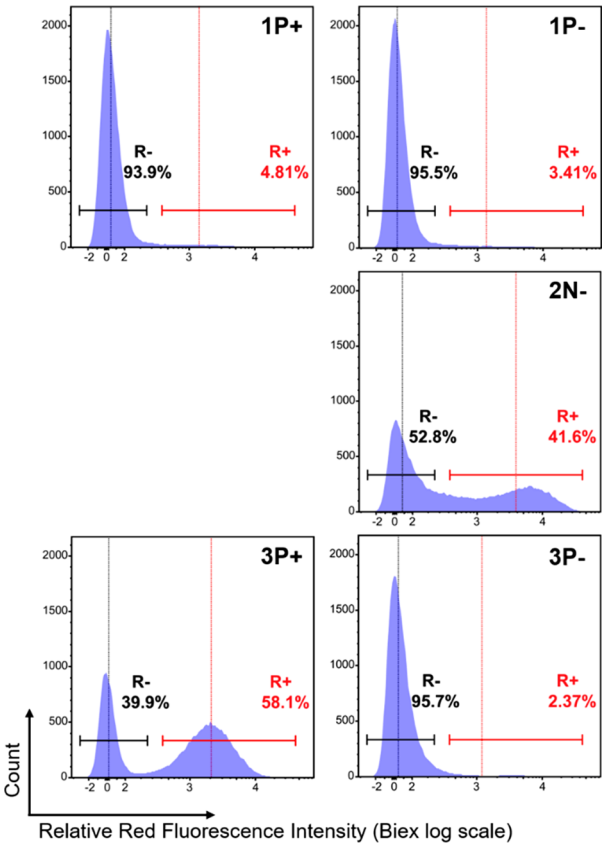

Figure 2. Histograms of three selection rounds to identify active SF_5PheRS enzymes via FACS cell sorting. Cells containing the whole library were cultured with 1 mM SF_5Phe (1P+) or without uAA (1P−). The RFP positive (R+) population in 1P+ (indicated by the red horizontal line) was selected for the next round of selection (2N−), which was conducted in the absence of SF_5Phe. The RFP negative (R−) population of 2N− (indicated by the black horizontal line) was collected and cultured with 1 mM SF_5Phe (3P+) or without SF_5Phe (3P−). The R+ population in 3P+ was sorted for further characterization. The black and red vertical lines identify the means of the R−/R+ populations.

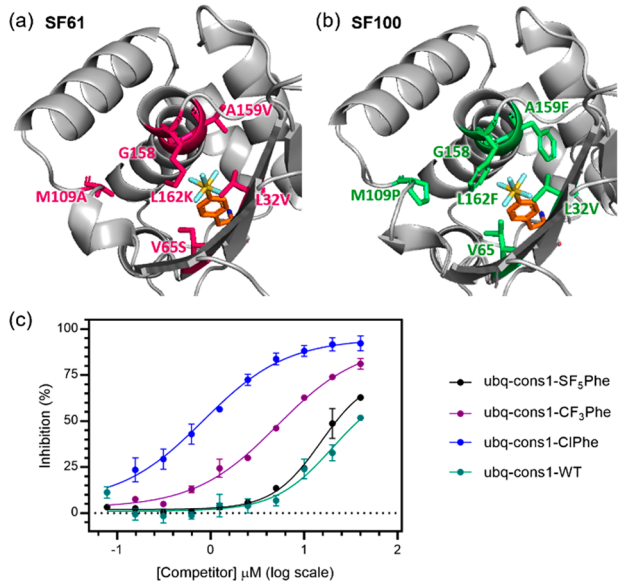

Figure 3. Rosetta-predicted structures of (a) SF61 and (b) SF100 with SF_5Phe as ligand. Mutation sites are highlighted in magenta and green. (c) Fluorescence polarization competition assay of Ubq-cons1 samples with either SF_5Phe, CF_3Phe, ClPhe, or Phe as the C-terminal residue (with Phe corresponding to the wild type). The standard error of duplicates or triplicates of each sample is shown by the error bar.

To illustrate the potential of SF_5Phe in the design of new protein interactions, we explored the potential of a Phe → SF_5Phe mutation to increase the binding affinity of the clamp binding motif (CBM) with the *E. coli* β-clamp protein. The interaction between CBM and sliding clamp is critical for nucleotide extension and proofreading exonuclease activity of Pol III and has been validated as an antibacterial drug target.[13−15] The consensus sequence QLDLF (cons1) is known to bind to the β-clamp with high affinity[16] and we probed this interaction further by replacing the C-terminal Phe residue by either SF_5Phe, *p*-trifluoromethyl-phenylalanine (CF_3Phe), or *p*-chloro-phenylalanine (ClPhe), which was previously reported to confer sub-μM binding.[17] A ubiquitin

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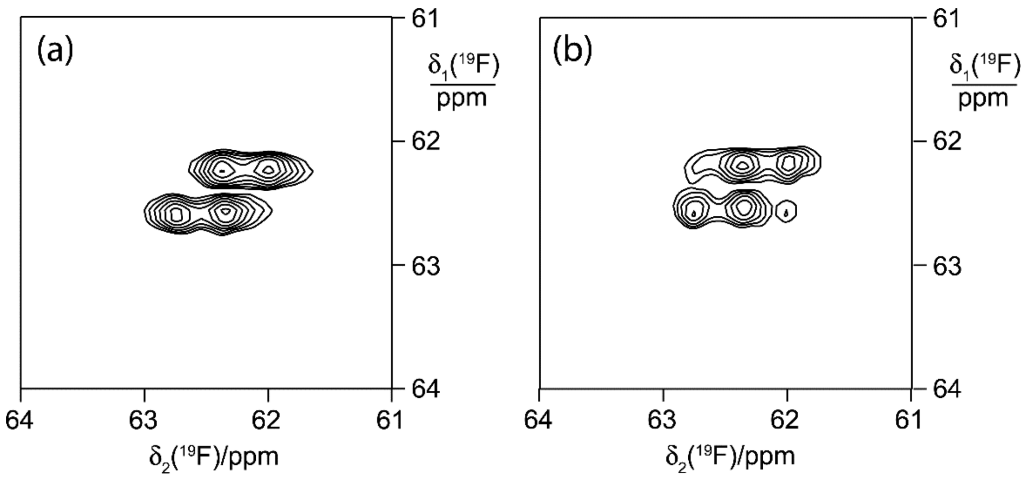

Figure 4. $^{19}F-^{19}F$ NOESY spectra of a 0.56 mM solution of the PpiB double-SF_5Phe mutant in 90% H_2O/10% D_2O, showing NOE cross-peaks between residues 27 and 98. The spectra were recorded on a 400 MHz NMR spectrometer using mixing times of (a) 200 μs and (b) 200 ms. The spectral region shown displays the diagonal peaks of the equatorial fluorine atoms of the SF_5 groups, which appear as doublets ($^2J_{FF}$ = 149 Hz) due to couplings with the axial ^{19}F spins. Splittings due to $^{19}F-^{19}F$ couplings were eliminated in the F_1 dimension by recording the experiment in a constant-time fashion (t_{1const} = 7 ms),[12] whereas the $^{19}F-^{19}F$ doublet fine-structure was retained in the F_2 dimension. Cross-peaks between the two sets of four equatorial ^{19}F spins were observable only when using the longer mixing time. The spectral region of the axial ^{19}F spins is not shown.

construct was designed, where the C-terminal glycine residue of ubiquitin was mutated to alanine and followed by the cons1 motif with the phenylalanine residue mutated by introduction of an amber codon (TAG), which was followed by the Mxe-intein[18] with C-terminal His_6-tag (Ubq-cons1-int). The corresponding pETMSCI-ubq-cons1-int plasmid (Figure S6) was used together with a modified pEVOLV vector[19] containing the SF61 gene and opt-*Mj*YtRNA (referred to as pEVOL-SFRS). SF61 proved to successfully incorporate also CF_3Phe (Figure S9). Co-transformation of *E. coli* BL21(DE3) with pEVOL-SFRS and pETMSCI-ubq-cons1-int and protein expression in the presence of 1 mM SF_5Phe or CF_3Phe yielded full-length Ubq-cons1-int samples, which were readily purified via Co^{2+} affinity chromatography and cleaved from the C-terminal Mxe-intein and His_6-tag by treatment with β-mercaptoethanol to generate the final Ubq-cons1 mutants. Similarly, pEVOL-CNRS encoding the polysubstrate-specific CNPheRS was used to incorporate ClPhe.[20] The identity of all protein products was confirmed by mass spectrometry (Figure S7). A fluorescence polarization competition assay[21] using these Ubq-cons1 mutants revealed that both ClPhe (IC_{50} = 0.8 μM) and CF_3Phe (IC_{50} = 4.3 μM) significantly increased the binding affinity to the sliding clamp, while SF_5Phe (IC_{50}= 15 μM) showed slightly higher affinity compared to the wild-type Phe (IC_{50} = 21 μM) (Figure 3c). Computational modeling indicated that the interaction of the C-terminal residue of the cons1 motif with the β-clamp is governed by hydrophobic contacts, with the binding pocket being close to electroneutral (Figure S8b). In view of the high electronegativity and relatively large volume of the SF_5 group, its binding affinity to the β-clamp could have been expected to be lower than that of ClPhe or CF_3Phe. Yet, it proved to be a better binder than phenylalanine in the tightly binding consensus CBM.

SF_5Phe's strongly hydrophobic and polar interactions also naturally lend themselves to site-specifically adhere a protein to membranes and other fluorophilic molecules. We employed β-cyclodextrin (βCD) as a probe. It is a well-studied model system for hydrophobic binding and is used in many applications from biosensing to protein delivery.[22−24] As expected, βCD strongly binds SF_5Phe as free amino acid (K_d = 830 μM; Figure S10c), whereas phenylalanine displays only weak binding. We then further expressed ubiquitin with SF_5Phe in the solvent exposed residue position Glu18 (yield 20.9 mg/L) and found it to bind βCD with even higher affinity (K_d = 107 μM; Figure S10a). In comparison, the same ubiquitin construct with CF_3Phe shows approximately 45 times weaker binding (K_d = 4.85 mM; Figure S10b), providing further evidence that SF5 is more than an expensive CF3 replacement.[25] Such high affinities for βCD are rivaled only by derivatives of adamantane.[26] The moderately hydrophobic adamantane moiety optimally fills the cavity of βCD and, to our knowledge, has not been genetically encoded to date.

Next we tested SF_5Phe as a structural probe in NMR spectroscopy. Two phenylalanine residues (Phe27, Phe98) in the structural core of PpiB point their side chains toward each other, bringing H^{ζ} atoms within 6 Å. We mutated both residues to SF_5Phe to study the NMR spectroscopic properties of SF_5 groups in proteins (Figure S11). The amber-interrupted PpiB gene was encoded by a pET3a vector. pEVOL-SFRS and pET3a-PpiB were cotransformed into *E. coli* BL21(DE3) and cultured with 2 mM SF_5Phe. The double-SF_5Phe-labeled PpiB mutant was produced with a yield of 30 mg of purified protein per liter of cell medium (Figure S12). The protein was folded despite the burial of the two bulky SF_5 groups in the core of the protein, suggesting that the space demand imposed by the SF_5 groups is energetically counterbalanced by favorable hydrophobic interactions between the SF_5 groups. The two SF_5 groups exhibited different chemical shifts in 1D ^{19}F-NMR spectra, with the ^{19}F-NMR signals of the four equatorial fluorine atoms split into a doublet (~62.3 ppm), and the axial fluorine into a quintet (~83.5 ppm; Figure S13). $^{19}F-^{19}F$ NOESY experiments conducted with mixing times of 200 μs and 200 ms displayed cross-peaks at the longer mixing time, which were absent when the mixing time was short (Figure 4). This demonstrates that $^{19}F-^{19}F$ NOEs allow direct confirmation of short contacts in medium-sized proteins without any other resonance assignments. To the best of our knowledge, this is the first observation of intramolecular $^{19}F-^{19}F$ NOEs in a protein.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

In summary, we demonstrated that the new unnatural amino acid SF_5Phe can be efficiently and site-specifically incorporated into proteins. It can be used as a ^{19}F-NMR probe and serves as a versatile hydrophobic binding moiety with high polarity and hydrophobicity. Functional SF_5Phe tRNA synthetase variants exhibit active sites complementary to the properties of SF_5Phe (Figure S14). Live/death screening, mass spectrometry analyses, and deep sequencing confirmed their remarkable activity, selectivity, and diversity in sequence, yielding an estimate of 1.4×10^4 active mutants (Figures S4 and S5). To our knowledge, such a high diversity of functional *Mj*TyrRS variants has never before been observed for any other unnatural amino acid, which highlights the exceptional versatility of the SF_5 group to form specific interactions. Considering the unique physicochemical properties of the SF_5 group, the capacity to genetically encode SF_5Phe in high yields adds an important tool for targeting protein−protein and protein−membrane interactions. In particular, the dichotomy of high polarity, which confers specific interactions and enhances water solubility, combined with superhydrophobicity, which supports membrane permeability and increases affinity to hydrophobic pockets, make SF_5Phe an attractive new building block for polypeptide-based pharmaceuticals and new materials. In addition, the site-specific introduction of the SF_5 group provides a useful ^{19}F-NMR probe for protein studies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c07976.

Detailed description of aminoacyl-tRNA synthetase screening procedure, expression and purification of SF_5-containing proteins and competition fluorescence polarization assay, figures of used plasmid constructs and characterization of SF_5-containing proteins by mass spectrometry and NMR spectroscopy, comparison of O-sulfo-tyrosine with SF_5Phe tRNA synthetase selection, deep sequencing analysis of selected SF_5PheRS (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

SF_5Phe	*p*-pentafluorosulfanyl-phenylalanine
CF_3Phe	*p*-trifluoromethyl phenylalanine
ClPhe	*p*-chloro-phenylalanine
uAA	unnatural amino acid
SF_5PheRS	SF5Phe tRNA synthetase
GFP	green fluorescent protein
RFP	red fluorescent protein
FACS	fluorescence-activated cell sorting
CBM	*β*-clamp binding motif
IC50	half maximal inhibitory concentration
*β*CD	*β*-cyclodextrin
PpiB	peptidyl-prolyl *cis*/*trans*-isomerase B
NOE	nuclear Overhauser effect
NMR	nuclear magnetic resonance.

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J. Am. Chem. Soc. XXXX, XXX, XXX−XXX