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Deciphering the Fluorine Code—The Many Hats Fluorine Wears in a Protein Environment

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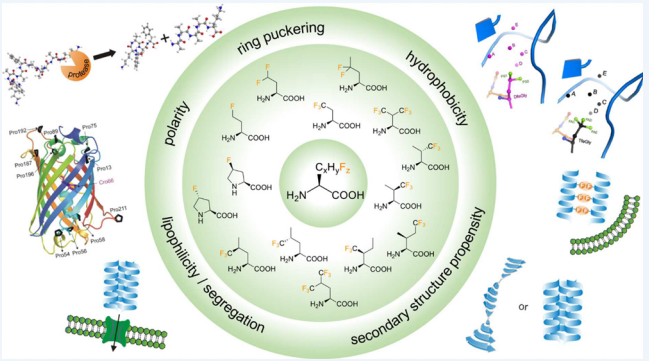
CONSPECTUS: Deciphering the fluorine code is how we describe not only the focus of this Account, but also the systematic approach to studying the impact of fluorine's incorporation on the properties of peptides and proteins used by our groups and others. The introduction of fluorine has been shown to impart favorable, but seldom predictable, properties to peptides and proteins, but up until about two decades ago the outcomes of fluorine modification of peptides and proteins were largely left to chance. Driven by the motivation to extend the application of the unique properties of the element fluorine from medicinal and agro chemistry to peptide and protein engineering we have established extensive research programs that enable the systematic investigation of effects that accompany the introduction of fluorine into this class of biopolymers. The introduction of fluorine into amino acids offers a universe of options for modifications with regard to number and position of fluorine substituents in the amino acid side chain. Moreover, it is important to emphasize that the consequences of incorporating the C−F bond into a biopolymer can be attributed to two distinct yet related phenomena: (i) the fluorine substituent can directly engage in intermolecular interactions with its environment and/or (ii) the other functional groups present in the molecule can be influenced by the electron withdrawing nature of this element (intramolecular) and in turn interact differently with their immediate environment (intermolecular). Based on our studies, we have shown that a change in number and/or position of as subtle as one single fluorine substituent has the power to considerably modify key properties of amino acids such as hydrophobicity, polarity, and secondary structure propensity. These properties are crucial factors in peptide and protein engineering, and thus, fluorinated amino acids can be applied to fine-tune properties such as protein folding, proteolytic stability, and protein−protein interactions provided we understand and become able to predict the outcome of a fluorine substitution in this context. With this Account, we attempt to analyze information we gained from our recent projects on how the nature of the fluorine atom and C−F bond influence four key properties of peptides and proteins: peptide folding, protein−protein interactions, ribosomal translation, and protease stability. These results impressively show why the introduction of fluorine creates a new class of amino acids with a repertoire of functionalities that is unique to the world of proteins and in some cases orthogonal to the set of canonical and natural amino acids. Our concluding statements aim to offer a few conserved design principles that have emerged from systematic studies over the last two decades; in this way, we hope to advance the field of peptide and protein engineering based on the judicious introduction of fluorinated building blocks.



INTRODUCTION

Of all the halogens, fluorine has the greatest abundance in the earth's crust, and even though inorganic fluorine can also be found in significant concentrations in marine and terrestrial organisms, no organofluorine compounds have ever been isolated from within the animal kingdom. From a few tropical plants and actinobacteria have been isolated fluoroacetate, fluoro fatty acids, fluoroacetone, fluorocitrate, a fluorinated nucleoside component of the antibiotic nucleosidin, and fluorothreonine.[1] As reviewed by Gouverneur et al. in 2008[2] and by Liu et al. in 2016,[3] fluorine has proven to be highly beneficial in the pharmaceutical and agrochemical industries, with over 150 fluorinated small molecules reaching the market since the 1950s and an increase, since 2010, from 20% to 30% of administered drugs containing fluorine atoms or fluoroalkyl groups. In addition, fluorine-18[4] and fluorine-19[5] are extraordinarily useful tools in medical imaging by means of positron emission tomography and magnetic resonance imaging, respectively.

In light of fluorine's impact on medicinal chemistry and drug development, it became obvious to introduce this unique element into biopolymers with the intention of improving or tuning their

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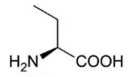
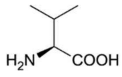
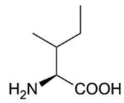
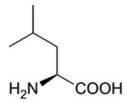
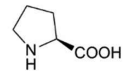
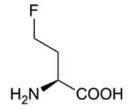
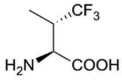
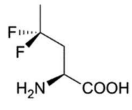
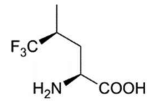
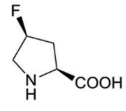
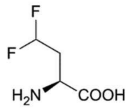
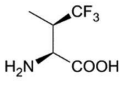
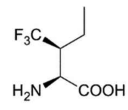
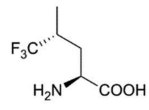
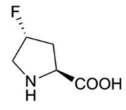
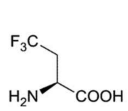
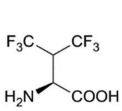
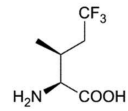
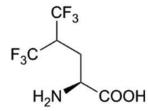
Table 1. Structures, Names, and "Three Letter Codes" for All Amino Acids Referred to in This Account[a]

hydrocarbon parent scaffolds				
				
Abu	**Val**	**Ile**	**Leu**	**Pro**
butanoic acid scaffold with gamma fluorination and no branching	*butanoic acid scaffold with gamma fluorination and beta branching*	*pentanoic acid scaffold with gamma or delta fluorination and no or beta branching*	*pentanoic acid scaffold with delta fluorination and gamma branching*	*4-fluoroprolines*
				
MfeGly	**(2*S*,3*S*)-TfVal**	**DfpGly**	**(2*S*,4*S*)-TfLeu**	**(4*S*)-FPro**
				
DfeGly	**(2*S*,3*R*)-TfVal**	**(2*S*,3*S*)-4'-TfIle**	**(2*S*,4*R*)-TfLeu**	**(4*R*)-FPro**
				
TfeGly	**HfVal**	**(2*S*,3*S*)-5-TfIle**	**HfLeu**	

[a]IUPAC names and common synonyms. Abu: (2*S*)-2-aminobutanoic acid, homoalanine; MfeGly: (2*S*)-2-amino-4-monofluorobutanoic acid, monofluoroethylglycine; DfeGly: (2*S*)-2-amino-4,4-difluorobutanoic acid, difluoroethylglycine; TfeGly: (2*S*)-2-amino-4,4,4-trifluorobutanoic acid, trifluoroethylglycine. Val: (2*S*)-2-amino-3-methylbutanoic acid, L-valine; (2*S*,3*S*)-TfVal: (2*S*,3*S*)-2-amino-4,4,4-trifluorobutanoic acid, (2*S*,3*S*)-trifluorovaline, TfV (diastereomeric mixtures); (2*S*,3*R*)-TfVal: (2*S*,3*R*)-2-amino-4,4,4-trifluorobutanoic acid, (2*S*,3*R*)-trifluorovaline, TfV (diastereomeric mixtures); HfVal: (2*S*)-2-amino-3-(trifluoromethyl)-4,4,4-trifluorobutanoic acid, (2*S*)-4,4,4,4′,4′,4′-hexafluorovaline, 4^3,$4'^3$-F_6Val, HfV; Ile: (2*S*,3*S*)-2-amino-3-methylpentanoic acid, L-isoleucine; DfpGly: (2*S*)-2-amino-4,4-difluoropentanoic acid, difluoropropylglycine; (2*S*,3*S*)-4′-TfIle: (2*S*,3*S*)-2-amino-3-(trifluoromethyl)pentanoic acid, $4'^3$-F_3Ile; (2*S*,3*S*)-5-TfIle: (2*S*,3*S*)-5,5,5-trifluoroisoleucine, 5^3-F_3Ile; Leu: (2*S*)-2-amino-4-methylpentanoic acid, L-leucine; (2*S*,4*S*)-TfLeu: (2*S*,4*S*)-2-amino-5,5,5-trifluoro-4-methylpentanoic acid, (4*S*)-trifluoroleucine, (4*S*)-5^3-F_3Leu, TfL (diastereomeric mixtures); (2*S*,4*R*)-TfLeu: (2*S*,4*R*)-2-amino-5,5,5-trifluoro-4-methylpentanoic acid, (4*R*)-trifluoroleucine, (4*R*)-5^3-F_3Leu, TfL (diastereomeric mixtures); HfLeu: (2*S*)-2-amino-5,5,5-trifluoro-4-trifluoromethyl pentanoic acid, (2*S*)-5,5,5,5′,5′,5′-hexafluoroleucine, 5^3,$5'^3$-F_6Leu, HfL; Pro: (2*S*)-pyrrolidine-2-carboxylic acid, L-proline; (4*S*)-FPro: (2*S*,4*S*)-4-fluoropyrrolidine-2-carboxylic acid, 4-*S*-Flp; (4*R*)-FPro: (2*S*,4*R*)-4-fluoropyrrolidine-2-carboxylic acid, 4-*R*-Flp

properties and facilitating structural studies.[6] Meanwhile, fluorine has been studied in the context of nucleic acids,[7] carbohydrates,[8] lipids,[9] and proteins, and the latter is the focus of this Account.

Fluorine has been shown to impart often favorable but seldom predictable properties to peptides and proteins, and the outcomes of such studies have been reviewed comprehensively elsewhere.[10,11] Recent reviews have also been written about the synthesis of fluorinated building blocks,[12] and strategies for their chemical or ribosomal incorporation.[13] Here we attempt to summarize how the nature of the fluorine atom and the C−F bond influence four key properties of peptides and proteins.

WHAT WE HAVE LEARNED OVER THE LAST TWO DECADES ABOUT FLUORINATED ANALOGUES OF NONPOLAR ALIPHATIC AMINO ACIDS AND PROLINE

In the early stages of the research programs of the Koksch and Budisa groups about 20 years ago, we were struck by how the introduction of fluorine into peptides and proteins and its impact on various properties of the resulting unnatural biopolymers could lead to such unpredictable outcomes. At that point in time, this unique element was far from being a suitable tool for rational design approaches. Thus, each of our working groups aims at understanding the molecular interactions that underly the impact of fluorination in the context of peptide and protein environments.

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The Koksch laboratory has systematically investigated fluorinated analogues of Abu (Table 1) in the context of peptide model systems that are designed to reconstitute natural protein environments. We have made the following observations: (1) small numbers of fluorine atoms introduce polar character into otherwise hydrophobic amino acid side chains, representing a departure from earlier studies that had demonstrated the hydrophobicity/lipophilicity of heavily fluorinated side chains; (2) fluorine dramatically changes the secondary structure propensity of aliphatic amino acids and, thus, the folding properties of accordingly modified peptides and proteins; (3) fluorine can considerably influence the proteolytic stability of peptides; and (4) the impact of fluorine not only depends on the nature of the fluorinated side chain but also on the immediate environment with which it interacts.

The Budisa group has focused on reprogramming protein translation in living cells for the efficient use of fluorinated amino acids as building blocks for the biological expression of proteins. Our particular aim is to understand the chemical reactivity and stereochemistry of the fluorinated building block in translation and its impact on the kinetics of protein folding.[14] Most recently, we have used fluorinated Pro (Table 1) as an excellent molecular model for this purpose, and it enabled us to make the following observations: (1) fluorinated Pro analogues dramatically increase biological expression of mussel proteins;[15] (2) the folding rate of globular proteins is affected not only by *cis*/*trans* isomerization but also the puckering of the proline ring;[16] (3) specific fluorine-tyrosine polar contacts can increase the stability of protein self-assembly;[17] and (4) there is a chiral bias with respect to fluorinated substrates for ribosomal synthesis, resulting in dramatic differences in the rates of peptide bond formation.[18]

We will show that the introduction of fluorine creates a fascinating class of amino acids with properties that are unique in the protein world.

THE NATURE OF THE C–F BOND AND HOW IT IMPACTS SOME PROPERTIES OF AMINO ACIDS

Fluorine is the most electronegative element, which means it has a large inductive effect and that the C–F bond is polarized with ionic character and a large dipole moment, facilitating electrostatic/dipolar interactions with its immediate environment. The three lone pairs of fluorine are tightly held, imparting the C–F bond with low polarizability, for example, making it a poor H-bond acceptor. The C–F bond possesses a low lying σ^*_{C-F} antibonding orbital as a consequence of the great extent of bond polarization; stereoelectronically aligned electron-rich bonds like C–H can thus donate electron density into this orbital to stabilize certain conformations via hyperconjugative interactions.[19]

How do the physicochemical properties of the C–F bond impact the measurable properties of amino acids, properties that in turn influence the characteristics of the peptides and proteins that contain them?

Hydrophobicity

From studies on the Abu, Val, Ile, and Leu scaffolds, we have learned that the volume and hydrophobicity of the side chain is influenced significantly by fluorination (Figure 1). In particular, MfeGly, DfeGly, and DfpGly are markedly polar due to the presence of highly polarized geminal and vicinal C–H bonds, as well as a strong fluorine-induced dipole moment within the side chain. Consider DfpGly, for which the van der Waals volume of the side chain is close to that of Leu but its hydrophobicity is lowered to a level slightly below that of Val.

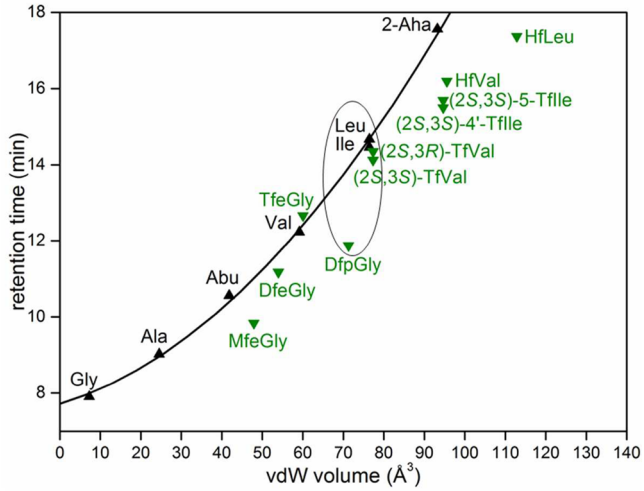

Figure 1. Relationship between van der Waals volume of the side chain and retention time in a reversed-phase HPLC assay. 2-Aha: (2*S*)-2-aminoheptanoic acid. The black oval highlights the fluorine-induced increase in polarity of DfpGly.

Secondary Structure Propensity

The intrinsic propensity of an amino acid for a certain secondary structure is an important property that must be considered in protein engineering.[20] Inspired by initial studies from the Cheng group[21] that we have expanded upon over time, we have learned that the number of fluorine atoms within an aliphatic side chain varies indirectly with the propensity for the peptide containing the given building block to adopt an α-helical secondary structure (Table 2). Only (2*S*,3*S*)-5-TfIle, with the highest helix propensity of all the trifluoroalkyl-bearing amino acids, bucks this trend.

Table 2. Helix Propensities for Selected Natural and Fluorinated Amino Acids

amino acid	helix propensity and literature reference
Ala	1.46 ± 0.01[22]
Abu	1.22 ± 0.14[26]
Leu	0.994 ± 0.093[22]
MfeGly	0.873 ± 0.068[27]
Ile	0.53 ± 0.05[23]
DfeGly	0.497 ± 0.060[27]
Val	0.41 ± 0.04[28]
(2*S*,3*S*)-5-TfIle	0.26 ± 0.03[24]
HfLeu	0.128 ± 0.023[26]
TfeGly	0.057 ± 0.022[27]
(2*S*,3*S*)-TfVal	0[28]
(2*S*,3*R*)-TfVal	0[28]
(2*S*,3*S*)-4′-TfIle	0[28]

Ring Puckering

Pro is unique among the 20 proteinogenic amino acids in that it possesses a pyrrolidine ring that spans the α-carbon (Cα) and nitrogen of the backbone and restricts the conformational space to distinct *cis* or *trans* states, for which there are characteristic torsion angles between Pro and the preceding residue. More than 90% of Pro in protein structures adopt the *trans* conformation, and the *cis*/*trans* conversion, requiring a 180° rotation about the peptide bond, is associated with a relatively high energetic barrier (~20 kcal/mol).[25] Wennemers and co-workers have extensively studied the conformational effects of a variety of ring substituents,

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including aminoproline, which provides a pH-triggered switch.[26] The introduction of fluorine at C-4 can be summarized as follows: (4*R*)-FPro strongly prefers the C$^{\gamma}$-*exo* ring pucker due to the gauche effect; the presence of fluorine reduces the energy barrier to isomerization by affecting the pyramidalization of the pyrrolidine nitrogen; thus, (4*R*)-FPro stabilizes the *trans* acyl-FPro conformation, while (4*S*)-FPro favors the *cis* conformation[27] (Figure 2). This demonstrates how targeted fluorination of

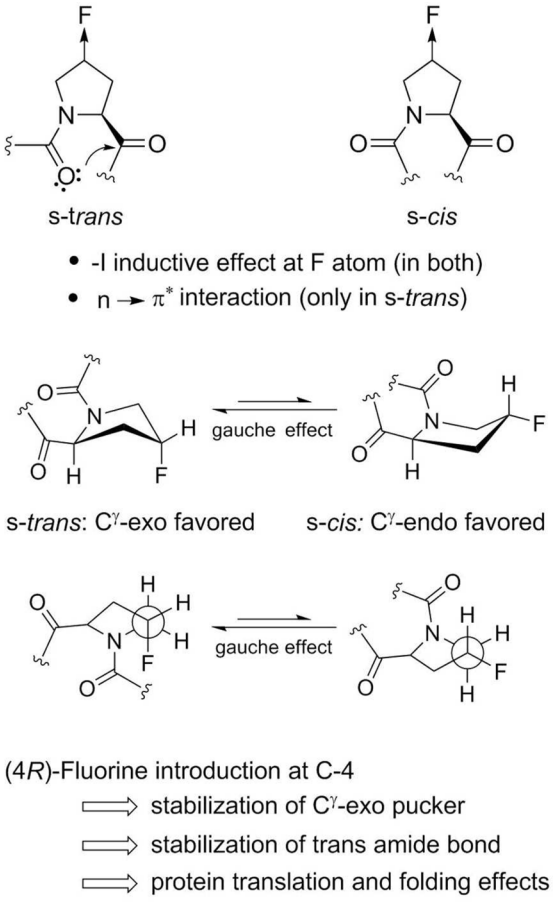

Figure 2. Schematic representation of the effects of fluorinating Pro at position 4 of its pyrrolidine ring. Complete discrimination between electronic and steric effects is impossible; thus, the term "stereoelectronic effect"[28] is used.

the Pro scaffold contributes to the elucidation of its roles in chemical or biological systems and offers new avenues for peptide and protein engineering.

PEPTIDE/PROTEIN FOLDING

α-Helical Coiled Coils

Coiled coils typically consist of two to seven right-handed α-helices that are wound around each other forming a left-handed superhelix. The primary structure is amphiphilic and can be characterized by a so-called heptad repeat $(abcdefg)_n$. Positions *a* and *d* are typically occupied by apolar residues (Leu, Ile, Val and Met) that form a hydrophobic core at the interface of the helices. By contrast, the positions *e* and *g* are frequently occupied by charged amino acids (most commonly Glu, Lys and Arg) that form interhelical electrostatic interactions. The remaining heptad repeat positions *b*, *c*, and *f* are exposed to the solvent and can in principle be occupied by any hydrophilic residue. Several prior studies had demonstrated that global substitution of hydrophobic core with highly fluorinated analogues of hydrophobic amino acids leads to increased thermal stability.[13] In contrast, work from our group revealed that single substitutions of minimally fluorinated building blocks within the hydrophobic core can lead to thermal destabilization. The degree of destabilization depends on how efficiently the fluorinated residue packs against neighboring side chains.[29−33] Of particular interest was the finding that the polarized β-methylene group of DfpGly influences the thermal stability of coiled coil dimers in a position-dependent way (Figure 3).

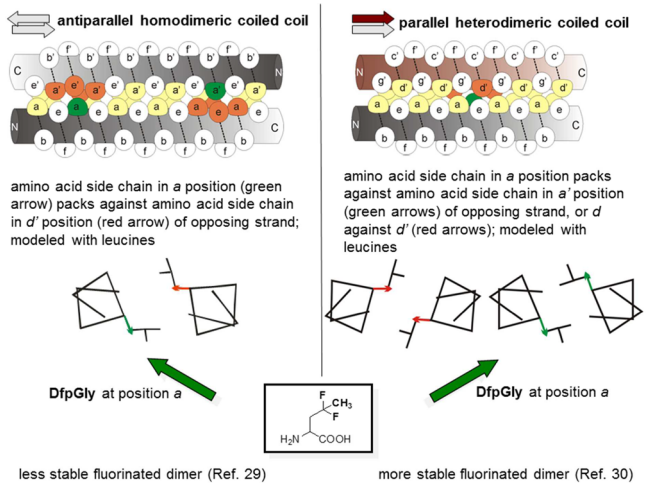

Figure 3. Impact of fluorine depends on its immediate packing environment. Adapted from ref 30 with permission from John Wiley and Sons.

To address the question of whether single substitutions with highly fluorinated side chains, thus borrowing from both extreme regimes described above, can restore or enhance the thermal stability of such structures, we studied the substitution of Leu with HfLeu, and of Val with TfVal in the heterodimeric parallel peptide model system referred to as VPE/VPK (Figure 4).[34] Whereas a higher thermal stability is observed for VPK containing HfLeu at position d_{19} (74.4 °C) in complex with VPE, compared to the parent helix bundle (70.7 °C), the melting points of VPK with (2*S*,3*R*)-TfVal or (2*S*,3*S*)-TfVal at position a_{16} were similar or lower, 70.8 and 67.5 °C, respectively. In this model system the central hydrophobic positions a'_{16} (Val), d'_{19} (Leu), and a'_{23} (Val) of VPE directly interact with the substituted position d_{19} of VPK. Therefore, the enhancement in the thermal stability for the HfLeu containing analogue is likely a result of greater hydrophobicity and efficient packing with the hydrocarbon side chains in the hydrophobic core, as had been previously demonstrated by our group by means of phage display experiments (Figure 5).[35] On the other hand, position a_{16} has d'_{12} (Leu), a'_{16} (Val), and d'_{19} (Leu) as its nearest neighbors and the shorter side chain of TfVal appears to contribute less to hydrophobic core formation.

Section Summary. The combination of hydrophobicity, polarization of neighboring groups, and the properties of the immediate environment determines the outcome of fluorine substitution within a hydrophobic protein environment.

β-Sheets

In collaboration with Czekelius[23,24] (2*S*,3*S*)-5-TfIle, (2*S*,3*S*)-TfVal, (2*S*,3*R*)-TfVal, and (2*S*,3*S*)-4′-TfIle were synthesized in their enantiomerically pure forms, incorporated into peptides,

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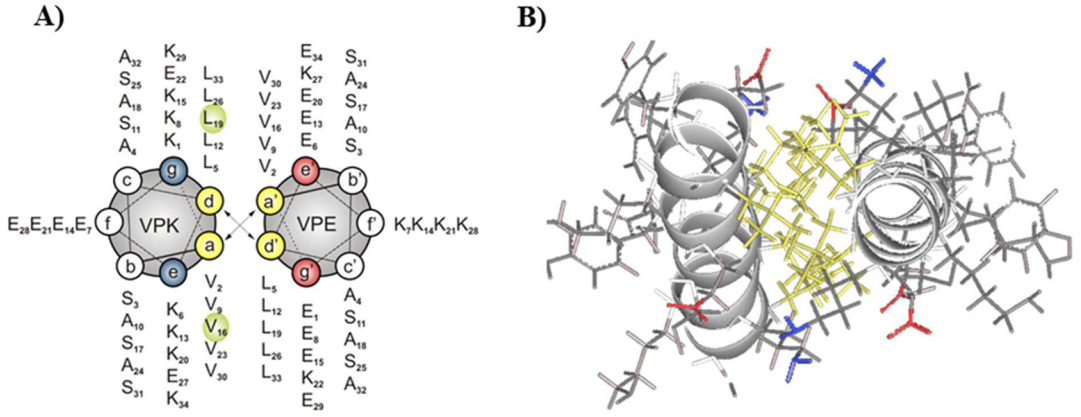

Figure 4. Parallel heterodimeric model system VPE/VPK as (A) helical wheel representation indicating Val16 and Leu19[34] and (B) ribbon and stick model.[36] Reproduced from refs 34 and 36 with permission from Elsevier and John Wiley and Sons, respectively.

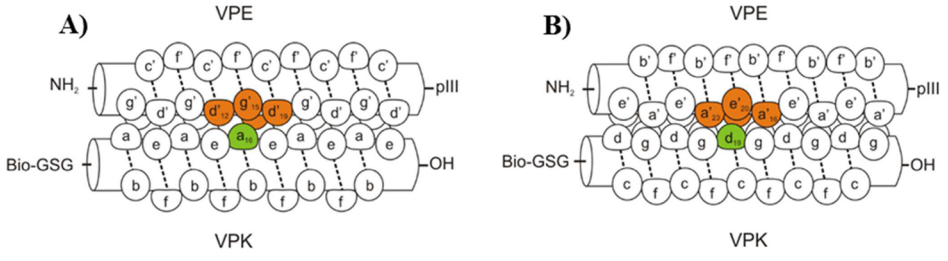

Figure 5. Cartoon showing packing interactions at positions (A) a_{16} and (B) d_{19} of VPE/VPK within the context of the phage display experiment.[35]

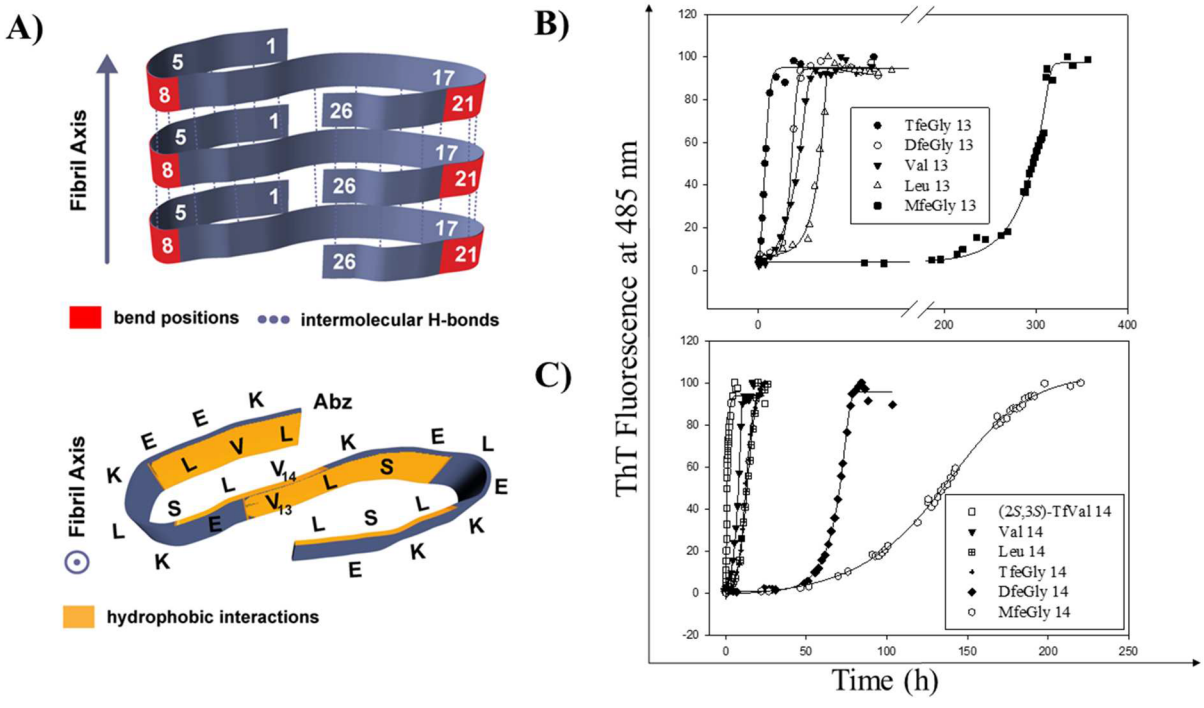

Figure 6. Effect of single substitution with fluorinated amino acids on the structural transition of VW18.[22] (A) internal fibril architecture and (B, C) ThT fluorescence at 485 nm versus time (h). Adapted from ref 22 with permission from The Royal Society of Chemistry.

and found to show extremely low α-helix propensities in all cases except (2*S*,3*S*)-5-TfIle (Table 2). Obviously, fluorination of isoleucine's methyl group at the β-branching point abolishes α-helix propensity, while fluorination of the δ-position rescues α-helix propensity to some extent.

We carried out a systematic study on the effect of fluorine on the rate of amyloid formation that took size, hydrophobicity, fluorine content, and secondary structure propensity of the building blocks into consideration.[22] We exploited a de novo designed model peptide (VW18) that adopts an α-helical coiled coil conformation upon dissolution in aqueous media and undergoes

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a time-dependent spontaneous structural transition into β-sheet rich amyloid fibrils.[37] Three judiciously placed valine residues, one at each an *f* (Val3), a *b* (Val13), and a *c* (Val14) solvent-exposed position of the α-helical coiled coil are responsible for this structural transition. Val13 was systematically replaced by MfeGly, DfeGly, TfeGly, and Leu, and Val14 was substituted by these residues in addition to (2*S*,3*S*)-TfVal and (2*S*,3*R*)-TfVal. The rate of amyloid formation, as determined in a standard ThT fluorescence assay, varies directly with the number of fluorine atoms at both positions 13 and 14 (Figure 6B and C).

Remarkably, both the Leu and MfeGly variants exhibit reduced amyloid formation rates compared to the more highly fluorinated species.

Section Summary. Secondary structure propensity is an important factor in amyloid formation kinetics, as is hydrophobicity. This observable is likely determined by the extent to which the initial helical coiled coil structure of the peptide is stabilized (Leu and MfeGly) or destabilized (TfeGly and TfVal). Finally, DfeGly exhibits the greatest position dependence, which may reflect the difference in polarity of the fibril environments of positions 13 and 14 (Figures 6A and 7).

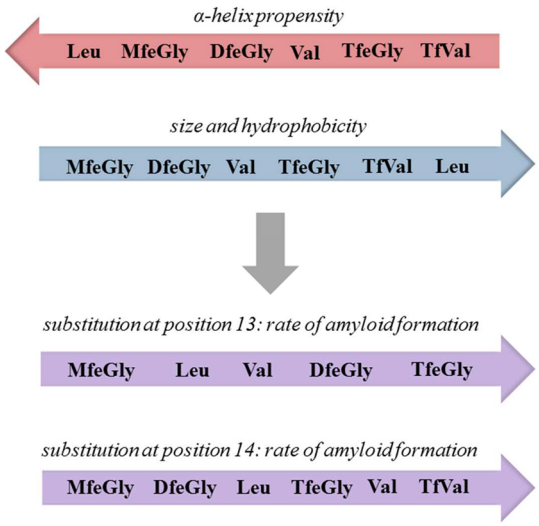

Figure 7. Schematic of the relationship between helix propensity, size/hydrophobicity, and rates of amyloid formation for VW18 with fluorinated aliphatic amino acids at position 13 or 14.

(4S)-FPro and (4R)-FPro in Enhanced Green Fluorescent Protein

Large globular protein folding requires escape from misfolded states caused by proline isomerization. The high barrier for isomerization results in characteristic refolding times of 10−1000 s at room temperature. For that reason, we were motivated to study and understand how Pro ring fluorination would influence these dynamics. To this end we solved the high-resolution crystal structure of enhanced green fluorescent protein (EGFP) with 10 Pro-residues globally replaced by (4*S*)-FPro or (4*R*)-FPro (Figure 8A).[16] Remarkably, EGFP containing (4*R*)-FPro was not expressed to a detectable level, whereas global substitution with (4*S*)-FPro yielded a soluble protein with faster refolding kinetics (Figure 8B).

Section Summary. Analysis of the structures revealed the following: (i) the majority of Pro residues in EGFP are involved in *trans* peptide bonds but a notable exception is Pro89 (Figure 8D); (ii) all Pros display Cγ-endo puckered pyrrolidine rings apart from Pro56, which adopts a Cγ-exo configuration; (iii) the fluorine atom in (4*S*)-FPro promotes *endo* puckering and thus preorganizes the pyrrolidine rings of 9 out of the 10 Pros into an optimal spatial arrangement, which is likely also responsible for the improved refolding properties. All of these effects result in 12 new fluorine-induced stabilizing interactions. Doubtless, the puckering of the pyrrolidine rings in the structure, which is dramatically influenced by fluorination, is the basic driving force behind the observed phenomena, also recently observed by Raines and co-workers in ribonuclease A.[38]

PROTEIN−PROTEIN INTERACTIONS

In an effort to address the question of fluorine's effect on protein−protein interactions in an otherwise well-characterized natural system, we substituted Lys15 of the bovine pancreatic trypsin inhibitor (BPTI) (Figure 9A), the residue that is part of the "interaction loop" and binds at the catalytic subsite S1 of the digestive serine protease trypsin (Figure 9B), with Abu, DfeGly, or TfeGly by solid-phase peptide synthesis and native chemical ligation.[39] Thermal denaturation studies under acidic conditions in the presence of either 6 M guanidinium chloride (GdmCl) or 8 M urea revealed that the Lys15Abu variant is significantly less stable than the native or fluorinated analogues (Figure 9C). Remarkably, the fluorinated side chains have a stabilizing effect that is greater even than that of the wild type in the presence of GdmCl, but the opposite is true in the presence of urea; this points to the fact that the positive formal charge of the solvent-exposed Lys contributes to the thermal stability of the natural inhibitor. Thus, DfeGly and TfeGly do not behave like canonical hydrophobic groups, which previous studies had found to be destabilizing at position 15.[40] Rather, the introduction of fluorine into the gamma methyl group facilitates electrostatic interactions with an aqueous environment due to the highly polarized nature of the C−F bond.

BPTI strongly inhibits trypsin by forming numerous contacts to the S1 subsite of the enzyme, and a water-mediated H-bond between the ammonium group of BPTI-Lys15 and Asp189 of β-trypsin plays an especially important role. We conducted standard inhibition assays with bovine β-trypsin[42] and found that substitution of Lys with Abu at P1 dramatically reduces inhibitor activity, whereas replacement with either DfeGly or TfeGly results in inhibitors that are as active as wild-type BPTI.

We determined high-resolution crystal structures of all BPTI variants in complex with bovine β-trypsin (note that we refer to the designated Protein Data Bank atom identifier labels in the following figures and text, for example, $3EG^{FAC}$ describes one of the gamma fluorine atoms (FAC) of residue TfeGly (3EG)). All complexes were superimposable, except at the P1 side chain and the water molecules within the S1 pocket. Analysis of the data revealed evidence for a "fluorophilic environment" for DfeGly and TfeGly within the S1 subsite of trypsin (Figure 10) based on multipolar interactions, as previously described by Diederich and co-workers for fluorinated small molecule drugs in complexes with enzymes.[43]

Interestingly, structural water molecule C is closer to fluorine atom $3EG^{FAC}$ in the TfeGly structure (3.4 Å) than it is to hydrogen atoms ABA^{HG3} in the Abu structure (3.6 Å) or OBF^{HG} in the DfeGly structure (3.7 Å) (Figure 11). Although it cannot be ruled out that this is a consequence of the slight shift in conformation within the side chain that occurs due to the electrostatic repulsion between $3EG^{FAC}$ and $3EG^{N}$, it may also indicate that a weak OH···FC H-bond exists between the TfeGly side chain and water C. Further investigation of this hypothesis

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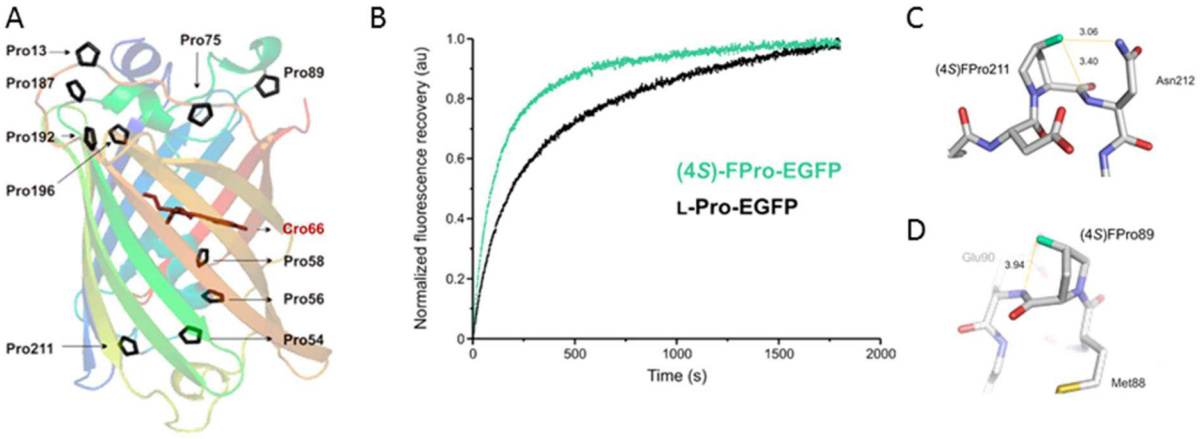
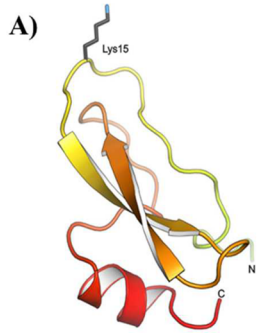


Figure 8. Role of fluorine-modulated ring puckering in the refolding kinetics of EGFP. (A) EGFP structure (PDB ID: 2Q6P) indicating Pro residues. (B) Refolding features of EGFP containing all L-Pros or all (4*S*)-FPros. Structural analysis enabled the mapping of novel fluorine interactions, exemplified by residues (C) 211 and (D) 89.[16]



C)

Residue 15	6 M GdmCl	8 M Urea
	T_M (°C)	T_M (°C)
Lys	66.5	75.8
Abu	63.4	71.7
DfeGly	68.8	74.8
TfeGly	68.3	73.8

Figure 9. (A) BPTI protein (PDB IDs: 3OTJ[41] and 2FTL[42]). (B) BPTI-trypsin complex with inhibitor in orange and enzyme in blue, black sphere represents position of Lys15. (C) Melting temperatures of BPTI variants.

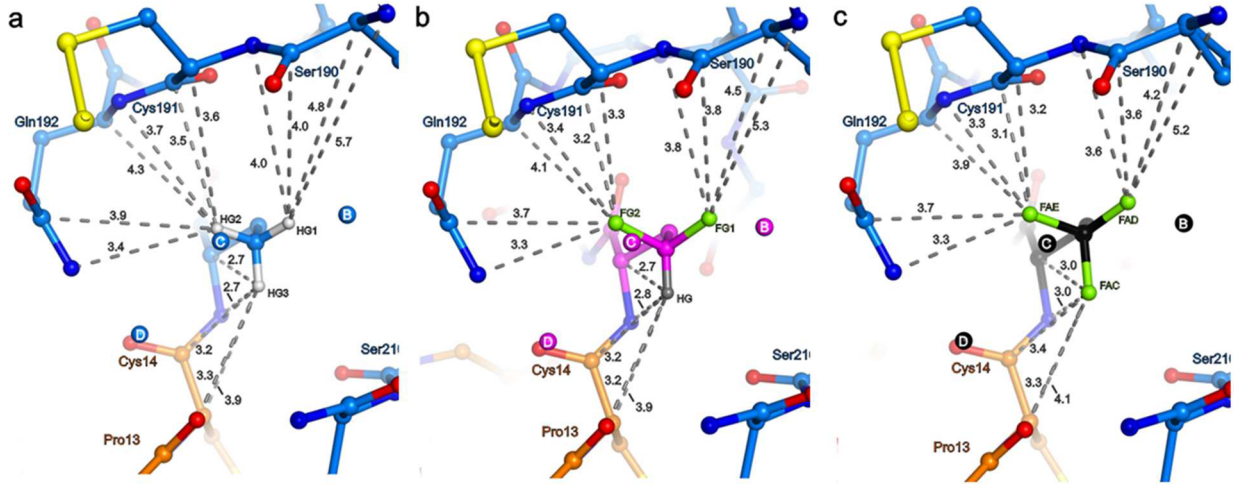


Figure 10. Distance analysis and top view of the unnatural side chain at position 15 of BPTI (main chain orange) within the S1 binding pocket of β-trypsin (main chain blue):[39] (a) Abu; (b) DfeGly; and (c) TfeGly. Reproduced from ref 39 with permission from The Royal Society of Chemistry.

would require neutron diffraction studies.[44] B-factor analysis for "new" waters B and C in the various structures provided further evidence to support this conclusion, namely that these values are significantly lower in the DfeGly and TfeGly complexes compared to Abu.

Section Summary

In contrast to nonpolar hydrocarbon amino acid side chains, fluorinated aliphatic groups can participate in multipolar and water-mediated interactions within the binding pockets of proteins and enzymes.

RIBOSOMAL TRANSLATION

Protein translation machinery naturally discriminates between unnatural/noncanonical amino acids and canonical building blocks via three key quality control mechanisms: (i) amino-acylation and editing of tRNAs by aminoacyl-tRNA synthetases

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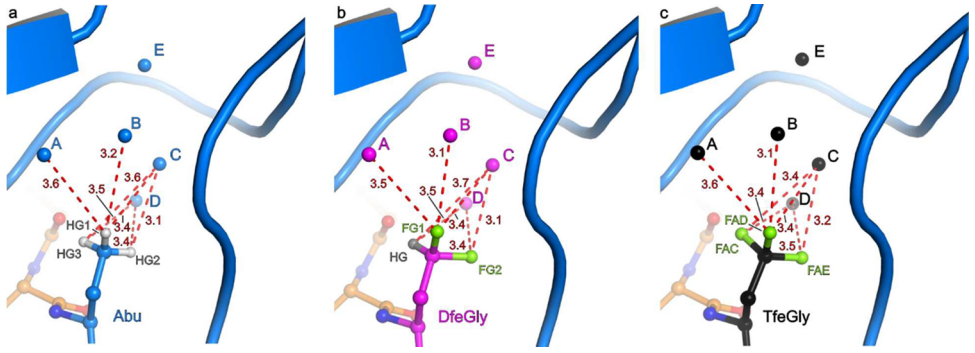

Figure 11. Water-mediated H-bond network around the unnatural side chain at position 15 of BPTI within the S1 binding pocket of β-trypsin.[39] Reproduced from ref 39 with permission from The Royal Society of Chemistry.

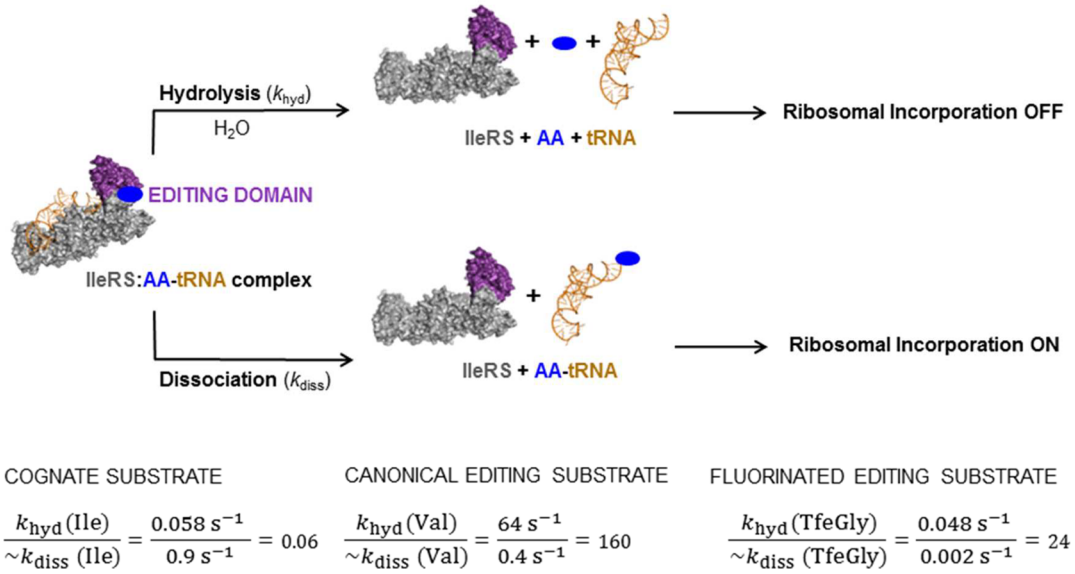

Figure 12. Ribosomal translation of TfeGly. Reprinted in part from ref 45. Copyright 2016 American Chemical Society.

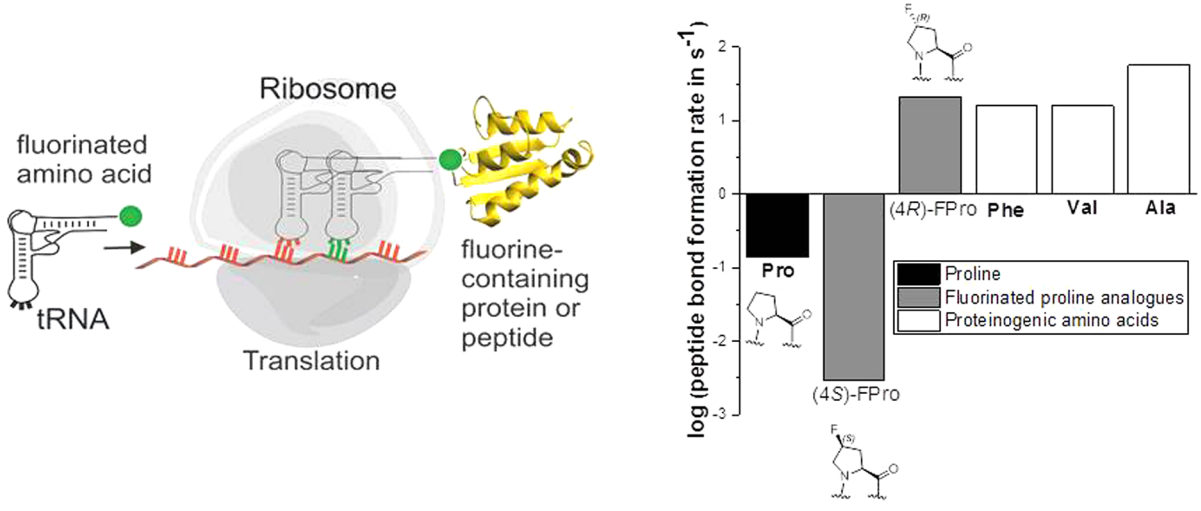

Figure 13. Ribosomal peptide bond formation with fluorinated Pro analogs reveals a chiral bias[15] toward (4*R*)-FPro. Note that ring pucker (directly influenced by fluorination, as shown in Figure 2) determines the rate of peptide bond formation.[18]

(aaRS), (ii) formation of ternary complexes with EF-Tu-GTP, and (iii) peptide-bond formation mediated by the ribosome. These mechanisms evolved in the presence of the natural canonical amino acid pool; however, because virtually all fluorinated amino acids are of chemical synthetic origin and were consequently absent during the evolution of these proofreading mechanisms, it has generally been assumed that translational quality control is not effective against these substrates. Whereas aminoacylation studies with fluorinated

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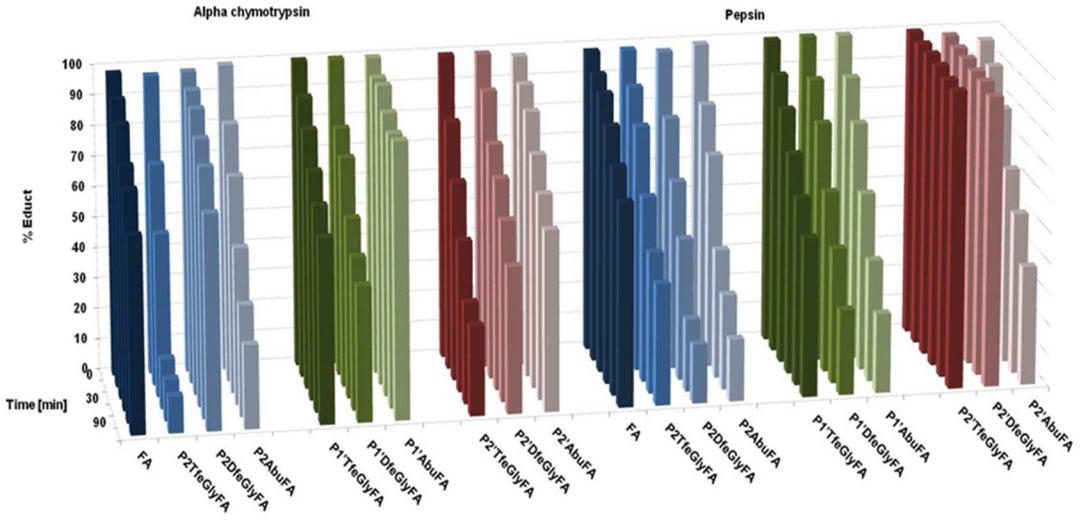

Figure 14. Digestion of peptide substrates by α-chymotrypsin or pepsin.

amino acids are decades old,[44] there has been a dearth of detailed mechanistic studies regarding their editing.

TfeGly

We reported recently that the editing domain of isoleucyl-tRNA synthetase (IleRS) is unable to discriminate between $tRNA^{Ile}$ charged with the cognate substrate Ile or the fluorinated amino acid TfeGly with respect to the hydrolysis rate (Figure 12).[45] Surprisingly, the competing reaction, the dissociation of TfeGly-$tRNA^{Ile}$ from the enzyme into solution for subsequent translation via the ribosome is also exceptionally slow, which had never been observed with amino acids that do not contain fluorine. As a result, hydrolysis outcompetes dissociation and the bioincorporation of TfeGly is not supported by the wild-type translation system. However, the first reported ribosomal translation of TfeGly was achieved by mutation of the enzyme's editing domain.

Section Summary. It is important to note that the kinetics of the individual processes comprising editing against TfeGly differ significantly from those found in editing against nonfluorinated substrates, which are characterized by much faster reaction rates (Figure 12). Obviously, the CF_3 group can engage in specific interactions with the synthetase.

FPro

Another factor that can potentially limit the production of fluorinated polypeptides is the rate of peptide bond formation within the ribosome. For example, peptide bond formation with Pro is exceptionally slow compared to all other proteinogenic amino acids, leading to ribosome stalling when a peptide sequence contains multiple such residues. A recent report showed that the introduction of a single fluorine atom into Pro to yield (4*R*)-FPro abolishes ribosome stalling by elevating the peptide bond formation rate to a level seen in the other proteinogenic amino acids (Figure 13).[18] Interestingly, its diastereomer (4*S*)-FPro produced the opposite effect.

Section Summary. Fluorine-induced effects on the stereoelectronics of the Pro pyrrolidine ring are fully reflected in the reaction rates that characterize ribosome-mediated peptide bond formation.

PROTEASE STABILITY

Peptide and protein-based therapeutics are characterized by their excellent selectivity compared to small molecules, but also by their low metabolic stability. Numerous reports have demonstrated that fluorinated building blocks can improve this property, though this is not always the case.[46,47]

To systematically investigate the influence of fluorinated analogues of Abu on stability toward protease degradation, the peptide sequence Abz-KAAFAAAAK (FA), was designed as a substrate with a central Phe residue to satisfy the known substrate specificities of serine and aspartic endopeptidases. The central portion of the FA sequence, -A-F-A-A-, can be written -P2-P1-P1′-P2′-. DfeGly, TfeGly, and Abu were substituted for alanine at positions P2, P1′, and P2′, and the variant sequences named accordingly. All peptide substrates were subjected to degradation by treatment with human blood plasma, elastase, α-chymotrypsin, and pepsin.[48,49] In the context of the blood plasma component elastase, TfeGly substitution immediately adjacent to the central Phe results in protease protection. For the digestive enzymes α-chymotrypsin and pepsin, the following fluorination patterns result in dramatic reductions in turnover: substitution at P2′ with either DfeGly or TfeGly in the pepsin substrate, and the presence of DfeGly at P2 in the α-chymotrypsin substrate (Figure 14).

Section Summary

In about 25% of all cases studied here, peptide substrates were protected due to the incorporation of a fluorinated amino acid.

GENERALIZATIONS THAT CAN OR CANNOT BE MADE, FUTURE DIRECTIONS, AND POTENTIAL APPLICATIONS

In a league of its own, fluorine enables protein engineering to achieve highly desirable outcomes, and we have come a long way in being able to predict them. For example, MfeGly has the highest helix propensity of the fluorinated amino acids described so far; thus, we feel confident in stating that it would support helical folding in a native protein environment. In contrast, adding one more fluorine substituent (DfeGly) may instead facilitate amyloid formation. Furthermore, the stereochemistry of a single fluorine substituent in a single Pro ring can dictate whether an unnatural protein product is expressed.

As has been known within the field of inorganic fluorine chemistry for many decades, where fluorine's reactivity and toxicity are major challenges, this element is a "diva" that must be handled with care. For the peptide community, the challenge in

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using fluorine as a tool lies in our ability to juggle the interplay between the specific properties of the fluorinated building block and its responsiveness to the environment it is exposed to. It is important to keep in mind that here are numerous entirely untapped aspects of the C−F bond that are waiting to be explored in a protein environment, including charge−dipole interactions and metal cation coordination.

Finally, from our vantage point, the next hurdle will be determining the way in which not just biomolecules in the laboratory, but whole living organisms accommodate fluorine. O'Hagan[50] and co-workers' groundbreaking publication about the fluorinase enzyme provided a major impulse for the detailed investigation of the effects of fluorine's incorporation into peptides and proteins. At this juncture, almost two decades later, the peptide community is ready to usher in a new era that will focus on the uptake, toxicity, and metabolism of organofluorine building blocks and that will be inspired by the kinds of systematic and interdisciplinary studies that we have reviewed here.

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Notes

The authors declare no competing financial interest.

Biographies

Allison Ann Berger received a B.A. degree from Reed College and a Ph.D. degree from TSRI La Jolla, both in Chemistry. After completing a two-year postdoctoral stay at the Max-Planck-Institute for Infection Biology as an Alexander von Humboldt fellow (2005−2007), she joined the group of Beate Koksch as staff scientist.

Jan-Stefan Völler studied chemistry at TU Berlin and received his Ph.D. degree in 2016 at Freie Universität Berlin in the group of Beate Koksch, working on a collaborative project with the group of Nediljko Budisa at TU Berlin. Currently, he is pursuing postdoctoral research in the Budisa lab on the ribosomal translation of novel fluorinated and other noncanonical amino acids.

Nediljko Budisa has been Professor of Biocatalysis at TU Berlin since 2010. He received his Ph.D. degree in 1997 and has done pioneering work in genetic-code engineering and most recently in chemical synthetic biology (xenobiology). His research in the context of fluorine biochemistry focuses primarily on the development of in vivo methods for introducing genetically encoded protein modifications in individual proteins, complex protein structures, and whole proteomes.

Beate Koksch received a Ph.D. degree from University Leipzig and pursued postdoctoral studies at TSRI La Jolla and postodoctoral lecture qualification at University Leipzig under Klaus Burger. She has been Professor of Chemistry at Freie Universität Berlin since 2004. Her group investigates fluorinated amino acids in the context of peptides and proteins, studies complex folding mechanisms in neurodegenerative diseases and develops new multivalent scaffolds.

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DEDICATION

Dedicated to the memory of Klaus Burger.

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