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Fluorine in Peptide Design and Protein Engineering

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The use of fluorine has become well established in the analysis of protein structure and function, e.g. in tools such as ^{19}F NMR spectroscopy. The application of the unique electronic properties of this element for the structural and chemical modification of peptides and proteins emerged as a promising approach. However, the influences of amino acid fluorination on side-chain interactions in proteins are still controversially discussed. The systematic investigation of the interaction properties of fluoroalkyl groups in a native polypeptide environment broadens the scope of fluorinated amino acids to the rational design of structural motifs and protein interfaces.

Introduction

The key to controlling the biological functionality and therapeutic efficacy of peptide-based drugs is provided by the proper amino acid composition and/or by the specific incorporation of building blocks that introduce the desired physical, chemical, and structural properties. However, the development of peptide-based drugs, on the basis of an alignment of the twenty canonical amino acids, is limited by profound factors such as weak metabolic stability, bad bioavailability in vivo, and insufficient biological potency. These issues arise from the limited variety of functional groups within the pool of proteinogenic amino acids as well as a high conformational flexibility of the peptide backbone.[1] The application of non-natural building blocks, which contain new functional groups such as heteroatoms that are not found in the natural amino-acid pool for peptide design and protein engineering, achieves strong and specific peptide-peptide or peptide-protein interactions.[2] Newly developed techniques such as solid-phase peptide synthesis (SPPS), enzymatic methods,[3] the methods of seg-

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Beate Koksch received her Diploma in Chemistry from University of Leipzig. In 1991 she joined the research group of Professor H.-D. Jakubke at the University of Leipzig where she earned her Ph.D. in Biochemistry in 1995. She was a DFG research fellow in the laboratories of Professor M. R. Ghadiri and Professor C.F. Barbas III at The Scripps Research Institute, La Jolla. In 2000 she returned to the University of Leipzig to start her independent career. In June 2004 she assumed a position as Professor of Organic Chemistry and Natural Products Chemistry at the Free University of Berlin. Her research interests focus on the interface of chemistry and biology and, in particular, on the role of complementary interactions and cooperativity on peptide and protein folding. Her laboratory has developed a screening system to investigate the interaction of fluorinated amino acids with native amino acids in a polypeptide environment. Other interests include the development of new methods for the protease-catalyzed incorporation of non-natural amino acids into peptides, the study of peptide-membrane interactions using ^{19}F NMR labeled peptides, as well as the evaluation of the potential of fluoroalkyl-substituted amino acids for peptide and protein modification using phage display.

MICROREVIEWS: This feature introduces the readers to the authors' research through a concise overview of the selected topic. Reference to important work from others in the field is included.

ment condensation,[4] native chemical peptide ligation,[5–7] expressed protein ligation,[8,9] and biosynthetic insertion via an aminoacylated suppressor-tRNA[10–13] extend the application of non-proteinogenic building blocks to proteins of higher molecular mass.

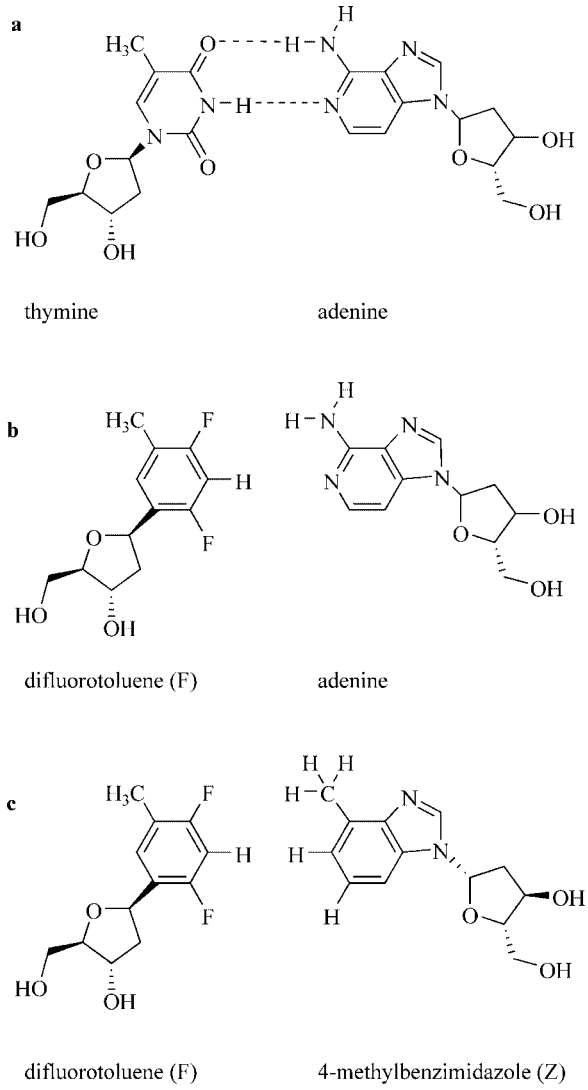
In this context, our work focuses on fluorinated amino acids. Fluorine, in fluorocarbon compounds, the so-called "organic fluorine", is beyond doubt an outstanding element in organic chemistry. Besides the synthetic potential of fluorine and of reagents derived from it, numerous fluorinated compounds, found from material sciences to medicinal chemistry, have great impact on our daily life. Although organic fluorine has proven to be valuable and auspicious in bioorganic and medicinal chemistry, it has attracted relatively little interest for peptide design and protein engineering. The main reason is the limited synthetic access to fluorinated building blocks and the elaborated methods that are often required for their incorporation. In drug design and development, fluorine is mostly used for the replacement of hydrogen,[14] e.g. in inhibitors and substrates of enzymatic reactions.[15] It has been shown that even just one fluorocarbon amino acid, which was incorporated instead of its hydrocarbon analogue, can have tremendous impact on protein structure.[16] However, the general consequences of incorporation of the fluorinated compounds, in particular, of the unique physical and chemical properties of fluorine, on the structure and activity of peptides and proteins are still far from understood. The impact of fluorine substitution on the properties of peptides and proteins has been shown to depend strongly on the position as well as on the extent of fluorine substitution within a certain amino acid or within the peptide chain, respectively. The class of fluorinated peptide building blocks is divided into two subclasses. One is formed by side-chain fluorinated amino acids, while the other subclass contains amino acids in which the C^{α} proton is substituted by a fluoroalkyl group. This difference not only dictates the properties exerted by the fluorinated building block but also the methods which have to be applied for amino acid synthesis as well as for their incorporation into peptides. Several aspects like the role of fluorine in hydrogen bonding, the space filling of fluoroalkyl groups and their interaction with hydrophobic domains are controversially discussed issues that remain to be studied systematically.

The scope of this review is to give an overview on achievements made in the area of peptide and protein modification by fluorine. It is our intention to focus on all aspects that play an important role in understanding the properties that fluorine develops within a polypeptide or protein environment.

I. Fluorine: The Unique Element in Organic and Medicinal Chemistry

The Role of Fluorine in Hydrogen Bonds

Undoubtedly, the ability to act as a hydrogen-bond acceptor is the most discussed issue of organic fluorine in literature.[17] Since hydrogen bonds are indispensable features in higher-ordered peptide and protein structure and, thus, provide a considerable contribution to the biological function of these macromolecules, this hard-argued aspect increases the value of fluorocarbon-modified amino acids for peptide design and protein engineering. Screenings of the Cambridge Crystallographic Structural Database and ab initio energetic calculations have led authors to the conclusion that organic fluorine is, if at all, a very weak hydrogen-bond acceptor.[18] According to these studies, C–F···H–C contacts represent the majority of contacts; interactions between carbon-bound fluorine and amine or hydroxy groups are not so common.[19] However, these contacts were denoted as weak polar interactions rather than hydrogen bonds because of their calculated weak energies. Carosati et al. recently performed an exhaustive analysis of the Protein Data Bank (PDB).[20] This study was done in order to determine the behavior of fluorine as a hydrogen-bond

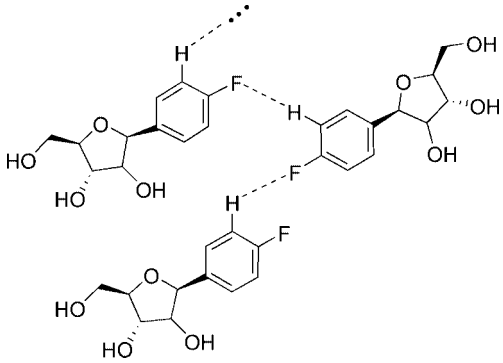


Scheme 1. Watson–Crick pairing of thymine and adenine (a) and base-pair stacking of adenine with the fluorinated thymine analogue (b) according to Diederichsen.[21] Interactions of both fluorinated nucleobase derivatives (c) according to Guckian et al.[24]

acceptor from protein–ligand complexes with cocrystallized fluorine-containing ligands. On the basis of the obtained statistical data, the authors characterized the fluorine-hydrogen-bonding geometry and developed a new angular function for fluorine, which has been shown to improve computational methods for the analysis of ligand–target complexes. It is further stated that fluorine-containing hydrogen-bonding interactions were observed in 18% of the investigated protein–ligand complexes.

The importance of hydrogen bonds in Watson–Crick base pairings for the fidelity of DNA polymerases in replication was studied by using thymine analogues with difluorotoluene as nucleoside.[21,22] The results have led to a new debate about the hydrogen-bond acceptor capability of fluorine. The authors of these studies describe the 2,4-difluorotoluene analogue to be apolar and unable to form the Watson–Crick like F···H–N hydrogen bond (Scheme 1a,b). Although this thymine mimic was shown to pair very poorly with adenine, it appeared to be a surprisingly good substrate for the polymerase – the enzyme efficiency is lowered only by a factor of 40. This led the authors to devaluate the contribution of hydrogen bonds to polymerase fidelity. In contrast, Evans and Seddon[23] interpreted these results the opposite way and considered the F···H–N contact to be strong enough to form a hydrogen bond. Guckian et al.[24] repeated these experiments with 2,4-difluorotoluene as a thymine analogue by using the nucleoside 4-methylbenzimidazole instead of adenosine as the counterpart for base pairing (Scheme 1c). In the resulting adenine analogue, the two nitrogen heteroatoms in the six-membered ring and the amino group, which is substituted by methyl, are missing. Again, this non-natural base pair has been shown to be replicated surprisingly well and selectively by DNA polymerase enzymes. According to the interpretation of Evans and Seddon, a C–F···H–C hydrogen bond must contribute to polymerase fidelity.

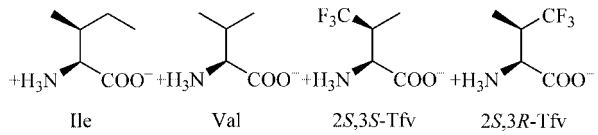
Parsch and Engels[25] reported C–F···H–C hydrogen bonds in RNA base pairs of fluorobenzene and fluorobenzimidazole nucleobase analogues, which were proved by crystal structures and calculations from thermodynamic data (Scheme 2). Thereafter, Kui et al. reported weak intramolecular C–F···H–C hydrogen bonds in polyolefin procatalysts.[26] Besides the role of organic fluorine as an acceptor in hydrogen bonds, they can act as hydrogen donors as well. This property has been shown for CF_2H groups; this effect is caused by an enhanced acidity of the attached proton.[27] In view of these results, the study from Caminati et al.,[28] which suggests that the difluoromethane dimer is stabilized by three C–F···H–C hydrogen–bonds, is not surprising.



Scheme 2. C–F···H–C hydrogen bonds in crystals of 1′-deoxy-1′-(4-fluorophenyl)-β-D-ribofuranose according to Parsch and Engels.[25]

The Steric Demand of Fluorocarbon Groups

Investigations on another contentious issue, namely the size of fluorine and fluorine-containing organic groups, have not yet resulted in a consistent concept. The van der Waals radius of fluorine (1.47 Å) is close to that of oxygen (1.57 Å) and is, thus, the next smallest atom after hydrogen, whose van der Waals radius is 1.2 Å.[29] As a result, fluorine is often used as an oxygen and hydroxy mimic in organic chemistry.[30] Interestingly, the steric demand of fluorocarbon groups seems to unproportionally increase with the extent of fluorine substitution. For example, the trifluoromethyl group is often described to be at least as large as isopropyl.[31] However, data of the exact size differ in the literature and depend mostly on the experimental methods from which they arise. One reason may be the difficulty to distinguish between the steric and electrostatic contribution of the fluorocarbon substituent on a chemical reaction and physical parameters, respectively.[29] Thus, the values of the molar volume of a trifluoromethyl group vary from twice the bulk of the methyl group,[32] as calculated from van der Waals radii, to even close to the size of phenyl and *tert*-butyl groups.[33] Experimental evidence for CF_3 to rather mimic bulkier alkyl groups within a protein environment was given recently by the work of Zanda on Tfm-containing protease inhibitors, which will be discussed in Section VI. A further indication that the trifluoromethyl group is a mimic of larger alkyl moieties rather than of the methyl group when interacting with proteins has been given by studies of the groups of Kumar and Tirrell.[34] They have shown that the 3*R*-form of 4,4,4-trifluorovaline (2*S*,3*R*-Tfv), which represents the diastereomer with the Tfm-group in the same orientation as that of the ethyl chain in Ile (Scheme 3), is accepted as a substrate for both the valyl- and the isoleucyl-tRNA synthetase. Thereby, the specificity constant for the valyl-tRNA synthetase was reduced by approximately 2500-fold, while the activation by the isoleucyl-tRNA synthetase was diminished by approximately 600-fold, each with respect to the activation of their native substrates Val and Ile, respectively. In contrast, (2*S*,3*S*)-Tfv, the diastereomeric form with the Tfm-group in the same orientation as that of the methyl group in Val and Ile, was not



Scheme 3. Amino acids used as substrates for valyl- and isoleucyl-tRNA synthetases.[34]

accepted by both enzymes. Thus, (2*S*,3*R*)-Tfv is a more efficient surrogate for isoleucine rather than for valine. Consequently, the trifluoromethyl moiety mimics the ethyl group better than the methyl group.

The Unique Electronic Properties of Organic Fluorine

Another valuable contribution to the special role of organic fluorine is provided by the unique properties of the C–F bond. Undoubtedly, this covalent fluorine–carbon assembly is special because of its hydrophobic character that is associated with a high dipole moment. This effect was recently described as polar hydrophobicity.[35] The origin of this unique property is, on the one hand, the high electronegativity of fluorine combined with a small van der Waals radius, which results in a short and strong covalent bond. On the other hand, the closeness of the outer electrons to the highly charged nucleus leads to a very low polarizability of the C–F bond. Fluorine has the smallest value of polarizability per molecular volume among the common elements found in organic compounds.[14] In addition, the C–F bond causes a reduced overall molecular polarizability throughout the carbon framework of the molecule. Therefore, fluorination of an organic compound leads to a general increase in lipophilicity.[35] However, it is also stated in the literature that terminal mono-, di-, or trifluorination of an alkane typically decreases the lipophilicity of the molecule,[31] although trifluorination of methyl groups is a widely applied approach in drug design to enhance the affinity of ligands for hydrophobic protein regions (see next chapter). In addition to polar interactions of carbon-bound fluorine such as C–F···C=O contacts,[36] the strong inductive effect of fluorine on specific heteroatoms can slightly decrease lipophilicity even in monofluorinated aromatic compounds. Polarity also strongly influences acidity or basicity of nearby functional groups, shifting the pK_a up to several log units.[37] These characteristics can be utilized by a directed incorporation of fluorine into bioactive compounds to create molecules with improved biochemical, physiological, and pharmacokinetic properties and thus obtain upgraded value for medicinal chemistry.[31]

The Advanced Role of Fluorine in Bioactive Compounds

The role of fluorine in medicinal chemistry has recently been reviewed by Böhm et al.[37] One eminent effect of the substitution of a carbon-bound hydrogen by fluorine is a tremendous enhancement of metabolic stability caused by the strength, and therefore, the chemical stability of the C–F bond.[38] This strategy has been applied in order to block oxidative metabolism of a drug by enzymes like cytochrome P450. Thereby, the rate, the route, and the extent of metabolic conversion can be influenced. Another beneficial aspect of this strategy is that it prevents formation of chemically reactive and toxic metabolites. A representative example is the fluorination of a potassium channel opener, a potential drug against some types of migraine headaches.[39] The non-substituted analogue has been shown to be hydroxylated at a phenyl carbon atom by a cytochrome P450 isomer. This leads to a metabolite that inhibits CYP450 activity. This undesirable impact causes serious drug–drug interaction. The substitution of the affected hydrogen by fluorine blocks this enzymatic conversion owing to the enhanced bond strength. In addition, the described modification, a substitution that causes minor impact on steric demand as well as on physicochemical properties of the compound, did not affect the oral bioavailability of the potent drug. Thus, a significant in vivo activity was observed. Another impact of fluorine on the bioavailability of a bioactive compound is the enhancement of its pharmacokinetic properties and of membrane binding and permeation. This effect can be achieved either by weakening the basicity of a functional group, for example a nitrogen atom,[40] due to the electron-withdrawing capability of fluorine,[37] or by a general increase in the lipophilicity of the molecule.[41] The ability to diffuse across membranes is indispensable for drugs that are supposed to have cerebral pharmacological effects, since they must pass through the blood–brain barrier. In studies with promazine, perazine, and perphenazine analogues, Gerebtzoff et al. have shown an enhancement of the membrane permeability coefficient by a factor of about 9 with the insertion of one trifluoromethyl group into the molecules.[41] The general increase in hydrophobicity through fluoroalkylation can provide a further advantage to drugs when binding to proteins as ligands. A lipophilic group attached to the ligand, which does not interfere with the active site of the targeted enzyme, enhances its bioactivity through binding to hydrophobic residues of the protein. Carbonic anhydrase inhibitors have been developed by using fluorocarbon lipophilic groups with larger hydrophobic surfaces than those of their hydrocarbon analogues.[42] The strategies described herein for the application of the special physico-chemical properties of organic fluorine in drug design and in the improvement of pharmacological parameters of bioactive compounds have led to the development of active agents for a wide variety of biochemical applications,[43] such as inhibitors of several enzymes, anticancer and antiviral agents, antibacterials, antidepressants and anorectic agents, hypolipidemic drugs and antidiabetics.[44]

II. Fluorine used as a Powerful Tool in Protein Analysis

^{19}F NMR Spectroscopy

Due to the fact that fluorine is usually not found in peptides and proteins, ^{19}F NMR spectroscopy has evolved into a sensitive and powerful tool in protein analysis, mainly because of the very low background signals combined with a high sensitivity (about 83% of ^{1}H NMR spectroscopy). A further advantage of this method is the relatively large range of chemical shift values, which gives access to the detection of even minor differences in the environment of

the ^{19}F label. As many proteins dramatically change their structural conformation when taken out of their native environment or are difficult to crystallize, ^{19}F NMR spectroscopic studies provide an important method, especially, for investigating membrane-associated proteins.[45] Furthermore, this analytical method provides access to proteins that are too large for conventional ^{1}H NMR studies. ^{19}FNMR spectroscopy is used to study protein conformations and their changes during folding and unfolding processes, and conformational changes because of solvent exposure and local mobility of specific regions and residues within the macromolecule, as well as the influence of ligand binding on protein structure. One way of labeling a protein directly is by incorporating fluorinated amino acids, commonly by using fluorotryptophan, fluorophenylalanine, and fluorotyrosine. These monofluoro-substituted derivatives are easy to synthesize, exert no enhanced steric demand relative to their native analogues, and thus do not cause perturbation of the protein structure. These building blocks can be incorporated biosynthetically using auxotrophic bacteria strains. The incorporation rates may differ over a wide range, depending on the fluorinated analogue to be inserted.[45] There is naturally no specificity for any single position in the protein. Thus, the amino acid will be substituted in each position it occurs naturally. The resulting resonances of the different labels have to be assigned unambiguously by site-directed mutagenesis. Shu and Frieden have used this strategy to study the unfolding process of a $(\beta/\alpha)_8$ protein by insertion of four 6-^{19}F-Trp residues that are all located in different elements of the secondary and tertiary structure.[46] The analysis of urea-induced unfolding gave insight into sequential denaturation and into the stability of the different structural elements of the barrel, and provides a basis for the investigation of the folding kinetics of this protein. The folding of chaperone PapD, which was studied by the same group,[47] was investigated with regard to the role of domain–domain interactions during the process of folding. In contrast to CD spectroscopy and fluorescence analysis, kinetic and equilibrium ^{19}F NMR spectroscopy of two 6-^{19}F-Trp residues indicated an intermediate folding state.

^{19}F NMR spectroscopy is also used for studying the properties of amino acid residues such as solvent accessibility and motional environment in so-called molten globule states.[48] In addition to exploring the structural properties of integrated membrane proteins, the interactions of amino acid side chains with lipid layers, which are involved in many biochemical processes and molecular assemblies, can be studied by using ^{19}F NMR spectroscopy. Grage and coworkers have used 5-^{19}F-Trp within the membrane-associated peptide gramicidin A to investigate the conformation and dynamics of the tryptophan side chain within a lipid environment.[49]

(αTfm)Ala was introduced into the peptaibol alamethicin by our group. Aib (aminoisobutyric acid, a non-fluorinated alanine analogue) at position 13 of alamethicin was substituted by (αTfm)Ala, thereby incorporating the fluorine label into the bend region of the alamethicin molecule, which is supposed to play a key role in the function of voltage-dependent ion channels.[50,51] Conformational analysis of the diastereomeric analogues by CD show that both diastereomers differ in their helicity and association properties, but one of the diastereomers exhibits the characteristic properties of alamethicin. In addition, single-channel measurements and microbiological tests show activities for the two diastereomers that are comparable to that of the native peptide.[52] Therefore, these fluorine-labeled alamethicin analogues are now used to study structure–function relationships of this membrane active molecule using ^{19}F NMR spectroscopy in combination with multidimensional NMR spectroscopic techniques and solid state NMR spectroscopy. This work was done in collaboration with the group of A. Ulrich.[53]

An advancement of the biosynthetic incorporation of fluorine-labeled tryptophan into proteins has been recently reported.[54] Instead of using protein expression in auxotrophic strains, which includes extra cloning and bears the risk of low yields and poor incorporation levels, the inhibition of tryptophan biosynthesis with 3-β-indoleacrylic acid was applied to accomplish the insertion of 5-^{19}F-Trp into the protein. Other methods use site-directed mutagenesis to place cysteine residues in appropriate positions of the protein followed by chemical loading of these side chains with a fluorine-containing label. Luchette et al. Cys-thioalkylated an integral membrane protein with 3-bromo-1,1,1-trifluoropropanone for structural studies after mutation of naturally occurring cysteines to alanine and incorporation of new Cys residues into selected positions.[55] Disulfide linkage of 2,2,2-trifluoroethanethiol (TET) to cysteine side chains for insertion of fluorolabels in vitro after protein biosynthesis was applied for studying the structural changes of mammalian rhodopsin during light activation by NMR spectroscopy.[56] In addition to applying fluorine-labeled aromatic amino acids, the incorporation of fluorinated analogues of aliphatic amino acids was reported.[57] Furthermore, the group of John F. Honek has focused on the biosynthetic incorporation of fluorinated methionine derivatives into bacteriophage λ lysozyme (LaL) in order to apply ^{19}F NMR spectroscopy to study the role of methionine in the structure and function of this enzyme. Although the substitution of 70% of all three methionines by trifluoromethionines did not influence the enzyme function, subtle conformational changes in protein structure caused by one fluorinated analogue were discussed.[58] Additionally, an inhibitory effect of this labeled building block on cell growth was observed. In further experiments, this group incorporated difluoromethionine into the same enzyme.[59] The diastereotopic properties of the two fluorine atoms were used to detect differences in rotational freedom of the methionine side chains that were located in solvent-exposed positions as well as within the tightly packed protein interior. In this case, however, it is questionable whether the rotational hindrance of the interior residue side chain observed is also present for the native methionine methyl group or if it is caused by the higher steric demand of the CHF_2 group in the substituted protein variant.

Another opportunity where ^{19}F NMR spectroscopy can be used for protein analysis is in the labeling of protein binding ligands. Doerr et al. investigated a hydrophobic binding site within a metal-assembled trimeric coiled coil protein using competitive titration studies of non-covalently bound hexafluorobenzene.[60] These experiments were used to study molecules that differ in hydrophobicity and in size, and helped to characterize the flexibility of the binding cavity, and enabled the authors to determine binding constants for a variety of compounds. Recently, libraries of fluorinated 2-pyridinone and amino acid derivatives were screened for their strength of binding to the chaperones PapD and FimC by using ^{19}F NMR spectroscopic techniques.[61] A comparison of the results with those obtained from surface plasmon resonance studies showed a general agreement of the two methods with only minor discrepancies. Furthermore, titrations of the fluorinated 2-pyridone library with a protein solution indicated the presence of multiple binding sites on PapD.

Another powerful method is one that uses rotational echo double resonance (REDOR) NMR spectroscopy for the solid state. This method allows the determination of internuclear distances between the nuclear spins of ^{19}F and other labels like ^{13}C, ^{15}N, and ^{31}P by measuring nuclear dipolar coupling constants. The ^{19}F-REDOR NMR applications include the fluorine-labeling techniques, which are used for solution NMR spectroscopy. For example, the fluorine-labeling of two Cys residues through disulfide linkage with 2,2,2-trifluoroethanethiol was used for structural investigations of light-activated, phosphorylated rhodopsin.[62] The REDOR NMR experiments of the ^{31}P-labeled phosphorylation site at the C-terminal tail and the fluorinated side chains within the protein provided information on the distances between the ^{31}P nucleus and the fluorine labels, and thus on a conformational change of the protein C-terminus during light activation. McDowell et al. labeled an inhibitor of human factor Xa, a serine protease that proteolytically converts prothrombin to thrombin during blood coagulation, with ^{13}C, ^{15}N, and ^{19}F in order to investigate its conformation within the inhibitor-protease complex using REDOR NMR spectroscopy.[63]

Raman Spectroscopy

A newly developing approach for the qualitative and quantitative analysis of fluoro-organic compounds that is based on Raman spectroscopy has recently been published.[64] Using a copper-vapor laser as light source, the excitation of fluorocarbon compounds at 510.8 nm results in specific emission bands in the range of 500–800 cm^{-1} for different types of carbon–fluorine bonds such as those in trifluoromethyl-, aromatic organofluorine-, and difluoromethylene groups.[65] This analytical method is useful for several biological applications. For example, the metabolic pathway of an administered drug containing at least one carbon–fluorine bond can be investigated using Raman spectroscopy. This strategy, which applies novel C–F Raman labels with specific fingerprints, is advantageous because of the chemically easy incorporation of the labels, a directly proportional dependence of the signal on analyte concentration, and a signal that is not affected by background interferences. These characteristics enable C–F Raman spectroscopy to play an important role in medical diagnostics, for example, in detection of cancer and pre-cancer conditions using fluorinated bioprobes such as proteins, nucleic acids, and drugs.

^{18}F Positron Emission Tomography

Positron Emission Tomography (PET) is a very common and powerful analytical method for medical diagnostics. ^{18}F is the most often applied radiolabel in such studies because of its low positron energy and the relatively long half-time of the isotope. The low emitting energy results in a marginal risk for the patient because of the short range as well as the limited dose of emission. The half-life of 110 min allows extensive and complex imaging protocols, including dynamic studies and investigations of slow metabolic processes. The application of fluorinated radiotracers, which include 2-[^{18}F]fluoro-2-deoxy-D-glucose, analogues of amino acids, peptide derivatives, fluorinated nucleosides, membrane phospholipids, and steroids, in positron emission tomographic studies for imaging in cancer patients has been recently reviewed.[66] Fluorinated analogues of L-Dopa have been used to probe the dopaminergic pathway in patients diagnosed with Parkinsons disease.[67] Interestingly, differences in the in vivo behavior of three ring-fluorinated isomers of L-DOPA, 2-[^{18}F]fluoro-L-DOPA, 5-[^{18}F]fluoro-L-DOPA, and 6-[^{18}F]fluoro-L-DOPA, have been observed.[68] These results indicate that minor chemical differences, such as the position of an aryl-fluorine substitution, can have a remarkable impact on pharmacological properties of drugs.

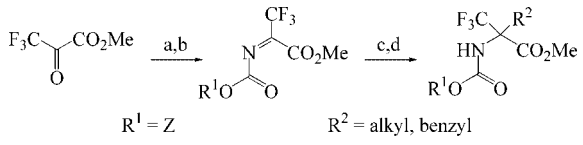
III. Synthesis of Fluorinated Amino Acids

Both the position as well as the extent of fluorine substitution within an amino acid dictates the methods that have to be used for the chemical synthesis of these building blocks. As these methods will be reviewed elsewhere soon, only general concepts will be mentioned here.

The synthesis of side-chain fluorinated amino acids either starts from malonic acid ester[69] or glycine,[70] or uses hexafluoroacetone as a protecting and activating agent.[71]

Several routes towards the synthesis of racemic $C^{\alpha,\alpha}$-fluoroalkyl-substituted amino acids have been developed.[72–79] Domino reactions starting from 5-fluoro-4-trifluoromethyloxazoles, including sequences such as nucleophilic substitution/Claisen rearrangement and nucleophilic substitution/benzyl group migration, provide ready access to racemic C^{α}-trifluoromethyl (αTfm) amino acids with side chains of high structural diversity.[80–86] The most general approach is the amidoalkylation of carbon nucleophiles

with alkyl-2-(alkoxycarbonylimino)-3,3,3-trifluoropropionates (Scheme 4).[87,88]



Scheme 4. Synthetic route towards fully protected C^{α}-fluoroalkyl-substituted amino acids.

IV. Enzymatic Resolution of Racemic Fluorinated Amino Acids

Considering the divergent biological activities of the enantiomers of fluoroalkyl-substituted amino acids and their diastereomeric peptide derivatives, the availability of these compounds in enantiomerically pure form is highly desirable. Most synthetic routes to optically pure amino acids rely on chemical[70,89–91] and enzymatic resolution.[92]

Enzymatic Resolution Using Esterase Activity

α-Chymotrypsin, a protease with esterase activity, is often used as a biocatalyst for this purpose, since it is easily and inexpensively available. This enzyme selectively catalyzes the hydrolysis of various esters of L-amino acids leaving the D-enantiomer unchanged. Although α-chymotrypsin preferably converts aromatic amino acids, amino acid esters with aliphatic side chains, as well as $C^{\alpha,\alpha}$-dialkylated and fluorinated[93] esters, have been shown to be substrates for enantioselective hydrolysis. The optical resolution and preparation of the L-enantiomers of various fluorine-substituted aromatic amino acids such as 2,3,4-fluorophenylalanines, 5-fluorotryptophan and 4-trifluoromethylphenylalanine have been accomplished by using this strategy. A lower hydrolysis rate was observed for the α-chymotrypsin-catalyzed resolution of $C^{\alpha,\alpha}$-dialkylated amino acids, in general, and α-methyl-4-fluorophenylalanine, in particular. Another protease, subtilisin Carlsberg, has been applied for the optical resolution of several fluorine-containing aromatic amino acids, for example, 3,4-fluoro-, 2,3,4,5,6-pentafluoro-, and 3,4-(trifluoromethyl)phenylalanine.[93]

It was reported by our group that proteases like subtilisin, α-chymotrypsin, or papain accept $C^{\alpha,\alpha}$-fluoroalkyl-substituted amino acid esters as substrates only to a very limited extent.[94] The application of these proteases for the optical resolution of racemic, fluorinated $C^{\alpha,\alpha}$-dialkylated amino acids is therefore excluded, except in the case for *Z*-(αTfm)Gly–OMe, which is a very good substrate for subtilisin.[95] A broad substrate specificity and enantioselectivity in the hydrolysis of esters with halogenated ester leaving groups can be achieved with lipases. This concept was used in the optical resolution of different monofluorophenylalanines by hydrolysis of their 2,2,2-trifluoroethyl or 2-chloroethyl esters.

Enzymatic Resolution Using Acylase Activity

Another approach is based on the enantioselective deacylation of *N*-acyl L-amino acid derivatives using acylases.[96] Keller and Hamilton have accomplished the optical resolution of *R*(+)2-trifluoromethylalanine by partial hydrolysis of the racemic *N*-trifluoroacetyl derivative with an enantioselectivity of 99.1% using hog kidney aminoacylase.[97] In contrast, experiments with 2-fluoromethylalanine revealed that the acylase was unable to discriminate between the enantiomers. Such aromatic and aliphatic *N*-trifluoroacetylated α-methyl amino acids are good substrates for the enantioselective deacylation catalyzed by carboxypeptidase A.[96] This enzyme has also been used for the optical resolution of several *N*-trifluoroacetylated fluorinated amino acids with high enantioselectivities such as monofluoro-, pentafluoro-, and trifluoromethylphenylalanines, and different monofluoro- and difluorotryptophans.[93] Although acylase I has been successfully used for the enantioselective conversion of *N*-acetylated derivatives of aromatic fluorinated amino acids, this enzyme is preferably used for the optical resolution of fluorinated aliphatic L-amino acids, for example, 4,4,4-trifluoroethylglycine, 6,6,6-trifluoronorleucine, 5,5,5-trifluoroleucine, and 5,5,5-trifluoronorvaline. In contrast, the deacylation of racemic 4,4,4,4′,4′,4′-hexafluorovaline with porcine renal acylase I failed, possibly because of the enhanced steric demand of the amino acid side chain within the enzyme binding pocket. One disadvantage of the *N*-deacylation method for the optical resolution of amino acids is the necessity to synthesize substrates that cannot be used as precursors for peptide synthesis. Besides additional synthetic steps, ion exchange chromatography must be used for the purification of the converted enantiomer.

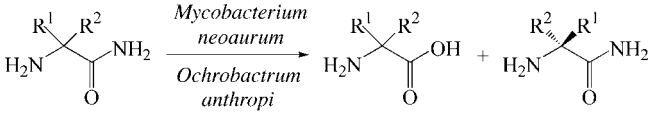
Table 1. Enzymatic resolutions of racemic $C^{\alpha,\alpha}$-fluoroalkyl amino acid amides using amidases from *Mycobacterium Neoaurum* and *Ochrobactrum Anthropi*.

	R^1	R^2	Enzyme Source	Conversion [%]	Amide *ee* [%]	Amino Acid *ee* [%]	*E* value[a]
1	CF_3 (Tfm)	CH_3	*Mycobacterium neoaurum*	47	98.0	96.0	>200
2	CF_2Cl	CH_3	*Mycobacterium neoaurum*	48	99.5	94.7	>200
3	CF_2Br	CH_3	*Mycobacterium neoaurum*	49	99.5	94.8	>200
6	CH_2Ph[b]	CF_2H (Dfm)	*Ochrobactrum anthropi*	58	98.7	67.9	25

[a] *E* values were calculated on the basis of the experimentally determined *ee* values of the amide and the acid. [b] This assignment is tentative.

Enzymatic Resolution Using Amidase Activity

Alternatively, $C^{\alpha,\alpha}$-dialkylated amino acid amides can be hydrolyzed enantioselectively with amidases.[96] DSM Pharma Chemicals (Geleen, The Netherlands) developed methodologies using amidase preparations of *Myobacterium neoaurum* ATCC 25795 and *Ochrobactrum anthropi* NCIMB 40321 (Scheme 5).[98,99] A technology that uses both the amidases described by Koksch et al. can be applied for the preparation of a variety of enantiopure $C^{\alpha,\alpha}$-fluoroalkyl-substituted amino acids.[100] Amidase from *Mycobacterium neoaurum* (ATCC 25795) hydrolyzes *R*,*S*-H-(αTfm)Ala-NH_2, *R*,*S*-H-(αCF_2Cl)Ala-NH_2, and *R*,*S*-H-(αCF_2Br)Ala-NH_2 with high enantioselectivities (E > 200) to give the pure *R*-amino acids. *Ochrobactrum anthropi* (NCIMB 40321) accepts *R*,*S*-H-(αDfm)Phe-NH_2 as substrate (Table 1).



Scheme 5. Enzymatic resolution of racemic C^{α}-fluoroalkyl amino acid amides.

V. Site-Specific Incorporation of $C^{\alpha,\alpha}$-Fluoroalkyl-Substituted Amino Acids into Peptides

A Special Focus on Protease Catalysis

In 2002, O'Hagan et al. reported the first native fluorination enzyme that biosynthesizes organofluorine metabolites.[101] This 5′-fluoro-5′-deoxyadenosine synthase from *Streptomyces cattleya* catalyzes the reaction of an inorganic fluoride ion and (*S*)-adenosyl-L-methionine. This reaction generates a C–F bond in 5′-fluoro-5′-deoxyadenosine (5′-FDA) with fluoroacetate and 4-fluorothreonine as secondary metabolites. The biosynthetic route towards fluoroacetate from inorganic fluoride has been reproduced in vitro with a cell-free extract from *Streptomyces cattleya*.[102] The enzyme has been isolated and purified[103] and its crystal structure has been recently reported.[104] Further investigations describe this enzymatic fluorination to follow an S_N2 reaction mechanism.[105]

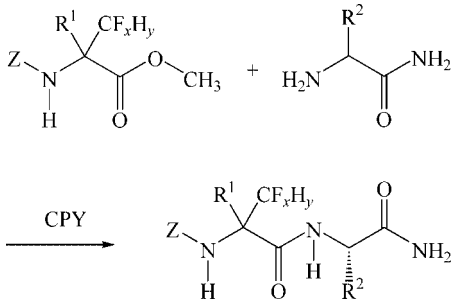
Several methods have been developed for the site-specific incorporation of fluorine-substituted amino acids in order to make the unique properties of fluorine available for protein and peptide chemistry. In addition to solid-phase peptide synthesis, monofluorinated surrogates of aromatic canonical amino acids and alkyl-fluorinated analogues of aliphatic residues can be inserted into peptides and proteins by biotechnological means using auxotrophic bacterial strains (see Sections II and VI). The incorporation of sterically and/or electronically demanding fluoroanalogues, however, needs alternative routes. More than 100 different non-natural amino acids, including several monofluorinated derivatives, have been biosynthetically incorporated into proteins by applying nonsense codon suppression methodologies.[106] This approach uses suppressor tRNAs that are aminoacylated with the non-natural amino acid of interest in cell-extract or cell-intact translation systems. The methodology is an excellent tool for the site-specific amino acid incorporation, covering a wide range of structural diversity. However, a considerable drawback of this method is the complexity of molecular biological, biochemical, and organochemical techniques that have to be applied.[106] Since relatively young techniques like native chemical ligation and expressed protein ligation (see ref.[5–7] and ref.,[8,9] respectively, for reviews) enable the assembly of proteins from altered peptide fragments, the insertion of challenging building blocks into polypeptide chains becomes increasingly interesting for protein engineering. The structure as well as the position and the content of fluorine within the amino acid determine the incorporation strategy. Side-chain fluorinated amino acids can be coupled by standard SPPS. $C^{\alpha,\alpha}$-fluoroalkyl amino acids demand elaborate solution methods.[83,85,86,107] Trifluoromethylation at the C^{α}-atom leads to a situation in which the high steric demand in combination with the strong electron-withdrawing effect drastically lowers the reactivity of the amino function.[108] H-(αTfm)Ala-OMe and several (αCF_2H), (αCF_2Cl), and (αCF_2Br) amino acids have been successfully incorporated into peptides by applying standard peptide coupling protocols.[94,109] The coupling of these amino acids to the peptide N-terminus can easily be accomplished with the reagent combination DIC/HOAt. The use of amino acid fluorides and chlorides and certain in situ coupling procedures give excellent yields for the incorporation of fluorinated amino acids at the C-terminus of the peptides.

An attractive alternative to classical peptide chemistry for the site-specific incorporation of non-natural amino acids into peptides and proteins is the combination of chemical and enzymatic methods. The valuable characteristics that enzymes provide are racemization-free catalysis under mild reaction conditions, high regio- and stereoselectivity, and the requirement of only minimal side-chain protection.[110–112] Successful enzymatic fragment condensations of both αTfm-containing acyl donors and -acyl acceptors with a variety of electrophilic or nucleophilic peptide fragments, respectively, have been achieved under catalysis with the proteases subtilisin, α-chymotrypsin, trypsin, and clostripain.[94,113,114] In all of those reactions, the efficiency of peptide bond formation depends on the position of the Tfm-substituted amino acid in the electrophilic and/or the nucleophilic fragments. However, the direct coupling of a Tfm amino acid to an electrophile with the above-mentioned proteases has not been achieved.

Peptide Bond Formation with α-Fluoroalkyl-Substituted Amino Acids Using Carboxypeptidase Y

In contrast, carboxypeptidase Y (CPY) is a protease that is known to feature high catalytic activity and enantioselectivity for kinetic resolution of several α-tertiary substi-

tuted carboxylic acid esters.[115] CPY has a broad S_1 and S_1' specificity and forms hydrophobic enzyme–substrate interactions.[116–118] We studied the applicability of CPY for peptide bond formation with $C^{\alpha,\alpha}$-fluoroalkyl-substituted alanine methyl esters as acyl donors (Scheme 6).[119]



Scheme 6. General scheme for CPY-catalyzed peptide synthesis.

Although more efficient in catalyzing peptide bond formation with the non-fluorinated carboxy component Aib, CPY can catalyze the direct coupling of (αTfm)Ala and (αDfm)Ala to various amino acid amides (Figure 1).

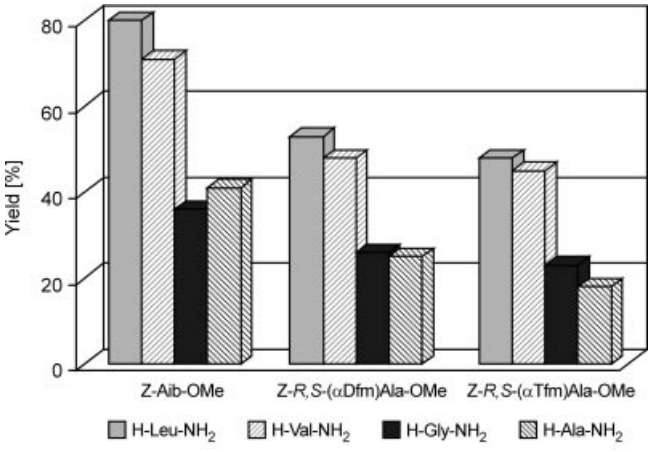


Figure 1. Results of CPY-catalyzed peptide synthesis using C^{α}-fluoroalkylated and methylated amino acids, and four different nucleophiles.[119]

In general, CPY accepts both enantiomers of (αTfm)Ala, while the *S*-configuration is always preferred in the P_1 position. These results suggest that the complete fluorination of a C^{α}-methyl group in Aib can result, depending upon the absolute configuration of (αTfm)Ala, in a stronger binding of the substrate within the active site of CPY. Interestingly, in the case of αTfm analogues, reactions of the enantiomer in which the bulkier trifluoromethyl group is in the same position as the α-proton in natural amino acids afford the peptide in higher yields. This implies that CPY accepts the Tfm group within the binding site for the α-proton. In this light, it will be very interesting to study the reaction behaviors of both enantiomers of (αDfm)Ala in CPY-catalyzed acyl transfers. The results of CPY-catalyzed peptide synthesis were the first examples of a direct enzymatic coupling of two different fluoroalkyl-substituted alanine derivatives to nucleophilic amino acid amides without any further activation of the carboxy components and without any medium or enzyme engineering. The use of amino acids[120] instead of their amides as amino components will extend the scope of this technique to the incorporation of a variety of fluorinated residues into biologically relevant peptides by enzymatic fragment condensation.

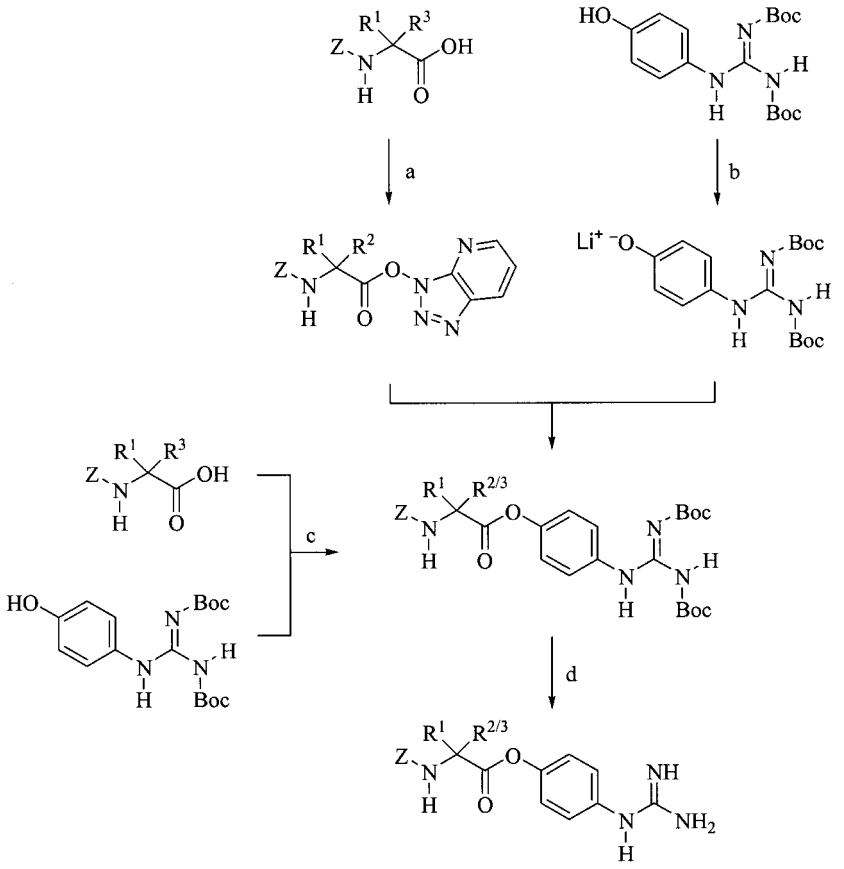
Substrate and Medium Engineering for the Protease-Catalyzed Incorporation of α-Fluoroalkyl-Substituted Amino Acids into Peptides

The direct enzymatic coupling of $C^{\alpha,\alpha}$-fluoroalkyl-substituted amino acids, however, is still a challenging topic because of the distinct substrate specificity of proteases. To overcome the limitations of the classical enzymatic approach, the powerful concept of substrate mimetics was developed.[121–126] The advantage of this concept is that the 4-guanidinophenyl ester (OGp) functionality mediates the acceptance of non-specific amino acid moieties in the specificity-determining S_1 position of Arg-specific proteases such as trypsin, thrombin, and clostripain, as well as of the Phe-specific protease α-chymotrypsin. The enzymatic incorporation of various sterically demanding $C^{\alpha,\alpha}$-fluoroalkyl-substituted amino acids into the P_1 position of peptides is possible with the use of this methodology.[127]

The 4-guanidinophenylesters of $C^{\alpha,\alpha}$-alkyl-substituted Ala derivatives can easily be prepared by reacting an N-protected amino acid with 4-[*N*′,*N*′′-bis(*tert*-butyloxycarbonyl)guanidine]phenol and by using TBTU as a coupling reagent. In contrast, the sterically more demanding $C^{\alpha,\alpha}$-alkyl-substituted Phe and Leu derivatives have to be activated with DIC/HOAt and reacted with the lithium salt of the guanidinophenol (Scheme 7), but can also be synthesized in high yields.

Several C^{α}-methyl and C^{α}-fluoromethyl-substituted amino acid guanidinophenylesters are accepted by trypsin as acyl donors and can be coupled directly to various nucleophiles of different length and sequence (Table 2). The yields of the peptide synthesis for αDfm-substituted amino acids are about twice as high as for αTfm and α-methyl-substituted derivatives. Product yields for the latter two derivatives were nevertheless found within the same range. These differences in synthesis efficiencies indicate a significant influence of the fluorinated $C^{\alpha,\alpha}$-substituent at the carboxy component on individual enzyme–substrate interactions. In order to further investigate this issue, we synthesized the dipeptides *Z*-(αDfm)Ala-Met-NH_2 and *Z*-(αTfm)Ala-Met-NH_2 by enzymatic acyl transfer reactions on a semi-preparative scale, separated the diastereomers using HPLC, and characterized all peptides by ^{19}F NMR spectroscopy.

A diastereomeric ratio of 1:3 was found for the α-trifluoromethyl-substituted dipeptides, which indicates that one enantiomer of the *Z*-(αTfm)Ala-enzyme complex is hydrolyzed faster by water than it is aminolyzed by the nucleophile. This consequently results in the release of the amino acid instead of the formation of a peptide bond. In contrast, the diastereomeric ratio for the α-difluoromethyl-



Scheme 7. Syntheses of $C^{\alpha,\alpha}$-dialkyl amino acid-4-guanidinophenyl esters. R^1: CH_3, CF_2H, CF_3; R^2: $CH_2C_6H_5$, $CH_2CH(CH_3)_2$; R^3: CH_3; a: DIC, HOAt, THF; b: *n*-butyllithium, THF; c: TBTU, DIEA, DMF; d: TFA, ultra sound.

Table 2. Yields (%) of trypsin-catalyzed peptide synthesis using substrate mimetics of $C^{\alpha,\alpha}$-dialkylated amino acids.

Acyl acceptor	Acyl donor: X_C^{α}:	*Z*-X_C^{α}Ala-OGp			*Z*-X_C^{α}Phe-OGp				*Z*-X_C^{α}Leu-OGp	
		Me	Dfm	Tfm	L-Me	D-Me	Dfm	Tfm	Dfm	Tfm
H–Gly–NH_2		18	35	14	16	50	47	40	58	13
H–Leu–NH_2		19	51	18	23	53	62	46	64	27
H–Met–NH_2		35	70	27	45	82	72	53	88	36
H–Ala–Ala–OH		20	65	22	41	68	87	63	90	40
H–Ala–Met–OH		25	62	25	28	56	82	47	88	48
H–Ala–Arg–OH		50	83	33	63	88	92	71	94	54
H–Ala–Ala–Lys–OH		22	51	17	47	77	79	53	83	37
H–Ala–Ala–Ala–OH		22	54	19	43	79	86	61	88	45
H–Ala–Ala–Pro–OH		37	64	28	54	86	87	61	90	41

substituted peptides was found to be 1:1. The observed identical product yields for both diastereomers give evidence that both enantiomers of the *Z*-(αDfm)Ala-enzyme complex are aminolyzed by the acyl acceptor at the same rate. Obviously, unlike the Tfm group, the Dfm group of at least one enantiomer interacts with the S′ region of the enzyme, and thus stabilizes the acyl enzyme intermediate. As a result, this additional enzyme–substrate interaction delays the reaction with any kind of nucleophile, including H_2O, and therefore leads to a simultaneous aminolysis of both enantiomers. Since the difluoromethyl group, unlike trifluoromethyl, is able to act as a hydrogen-bond donor,[27,28] the described stabilizing interactions appear to be of a polar nature. This influence of the absolute configuration of the αTfm-substituted Ala as an acyl donor ester on enzyme activity was shown to be of even higher importance when frozen aqueous reaction conditions[128] were used (Figure 2).

VI. Fluorine in Bioactive Peptides: The Impact on Biological Activity and Proteolytic Stability

Fluorine Modification of Bioactive Peptides

A strategy that exploits the steric and electronic properties of fluorine to improve the properties of biologically active peptides is the incorporation of fluorinated amide bond isosteres that mimic the peptide linkage. In addition

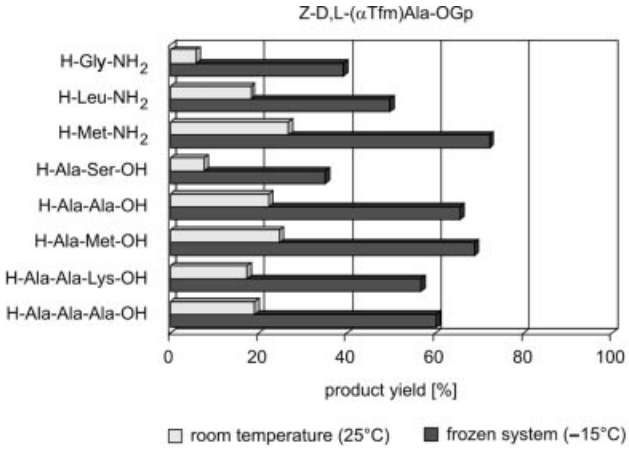
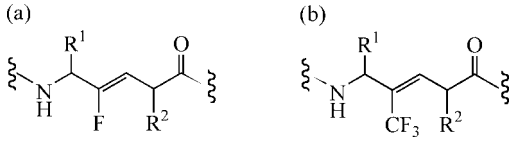


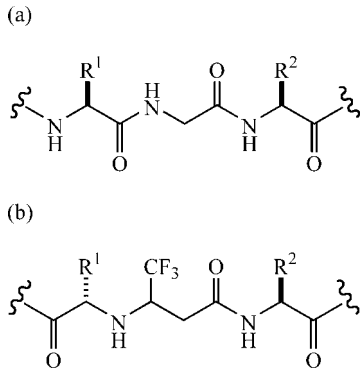
Figure 2. Product yields for trypsin-catalyzed substrate-mimetic-mediated peptide synthesis at room temperature and in the frozen aqueous system.

to an improved resistance towards proteolysis, fluorination of such moieties can lead to a better matching of steric demands and to an alteration of the electronic properties of the amide group. For example, the replacement of the amide group by a fluoroalkene moiety (Scheme 8a) in tripeptides led to inhibitors of the zinc peptidase thermolysin that bind one order of magnitude more tightly than the unmodified enzyme substrates.[129] Calculations on dipole moments show that the (trifluoromethyl)alkene isostere (Scheme 8b) represents an even better electrostatic mimic of the amide bond relative to the monofluoro-substituted alkene moiety.[130] The enhancement in backbone rigidity relative to the non-substituted alkene peptide bond isostere was shown by X-ray structural analysis.



Scheme 8. Fluoroalkene amide bond isosteres. Ref.[129] (a); ref.[130] (b).

Studies on peptidomimetics containing the conformational constraining trifluoromethyl group were recently published by Zanda.[131] These partially modified retro (PMR) ψ[NHCH(CF_3)]Gly peptides (Scheme 9b) adopt stable turn-like conformations mainly owing to the torsional restrictions caused by the stereoelectronically demanding trifluoromethyl group. Another approach toward fluorine-containing peptides and proteins uses side-chain fluorinated building blocks. Fluorinated amino acids, especially α-fluoromethyl-substituted and trifluoromethylated derivatives, possess valuable biological activities as has been demonstrated, for example, for several enzyme inhibitors.[132] Several bioactive peptides have been modified using fluorinated analogues of native amino acids, and their impact on the biological activity of the biopolymer was studied.[133]



Scheme 9. Standard (–Xaa–Gly–Xaa–) peptide (a) and partially modified retro ψ[NHCH(CF_3)]Gly peptide (b).[131]

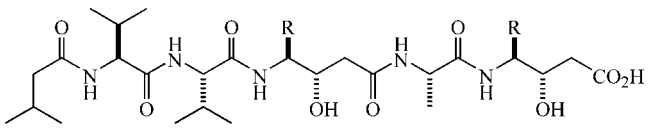
The strategies most used for amino-acid substitution involve the replacement of a hydrogen atom and a hydroxy group in aromatic amino acids by one fluorine atom or the fluorination of alkyl groups in aliphatic amino acids.

The replacement of the non-canonical amino acid 4(*R*)-hydroxyproline by (*R*)-4-fluoroproline in collagen analogues is a demonstrative example for the substitution of a hydroxy group in peptide building blocks by a single fluorine atom.[134] The studies on this topic, which have recently been reviewed by Yoder and Kumar,[135] point out a remarkable stabilization of the collagen triple helix by this alteration.

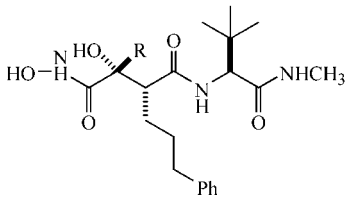
Directed modification of biologically active peptide building blocks with trifluoroalkyl groups to improve functional properties was successfully performed by Zanda.[131] In order to verify the isosteric properties of the Tfm-group, pepstatin A, a subnanomolar inhibitor of many aspartyl proteases, was modified by substituting the *iso*-butyl groups of both the statine residues with trifluoromethyl groups (Scheme 10a). The fluorinated analogue showed an only three times lower potential in inhibition of plasmepsin II, an aspartic protease from a malaria-causing protozoal, than that of the natural molecule. Furthermore, a decrease in inhibitory activity of pepstatin A towards human cathepsin D by the above-mentioned substitution was observed. This selectivity, obtained by fluoroalkyl substitution, makes the peptide analogue very attractive for malaria therapy. Crystal structures of plasmepsin II complexed with pepstatin A and the Tfm-pepstatin, respectively, exhibited almost identical conformations of the enzyme inhibitors, and thus prove that the trifluoromethyl group is a very effective mimic of an isobutyl in peptide/protein interactions. This finding was also confirmed by further studies of this group on peptidomimetic hydroxamate inhibitors of matrix metalloproteinases (MMPs).[131] The replacement of CH_3 by CF_3 in the quarternary α-methyl alcohol moiety of an MMP inhibitor (Scheme 10b) led to a dramatic drop in inhibitory activity toward different MMPs. One explanation for these results is the unsatisfactory fit of the Tfm-group into the binding pocket of the enzymes caused by the enhanced steric demand. The altered electronic properties of the trifluoromethyl group relative to a methyl group of the

original peptidomimetic protease inhibitor may contribute to this phenomenon as well.

(a)



(b)



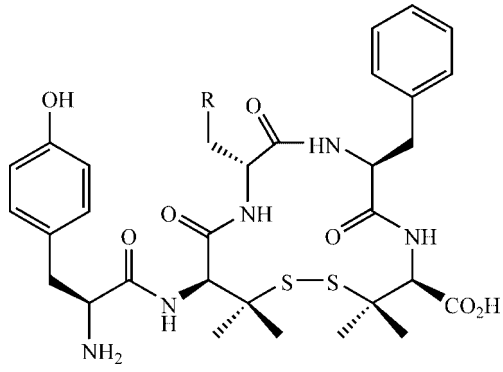
Scheme 10. a) Pepstatin A (R = isobutyl) and its bis-Tfm-analogue (R = CF_3); b) potent MMP inhibitor (R = methyl) and its Tfm-analogue (R = CF_3).[131]

In the last five years, several laboratories have focused their investigations on fluorinated analogues of hydrophobic canonical amino acids such as leucine and valine. These studies principally focused on the influence exerted by the fluorinated derivatives on structural stability as well as on biological activity of larger bioactive peptides and proteins. In this context, attention is focused on the nature of the "fluorine-effect" on hydrophobic interactions of protein interfaces. Horng and Raleigh have described two variants of an α-β globular protein, each modified by a single valine replacement to a 4,4,4-trifluorovaline within largely buried amino acid positions.[136] Both single amino acid modifications caused significant stabilization of the protein structure, as well as faster folding and slower unfolding processes by enhanced hydrophobic interactions. This example demonstrates the considerable impact that the fluorination of one single methyl group can exert on protein folding. Fluorinated analogues of melittin, a 26 amino acid residue amphiphilic peptide from bee venom, were synthesized by Niemz and Tirrell.[137] Substitutions of leucine residues by 5,5,5-trifluoroleucine increased self-association of the peptide in aqueous medium and membrane affinity. The extent of both effects has been shown to depend on the substitution position, the number of amino acids replaced, and the stereochemistry of the incorporated trifluoroleucine. Interestingly, the authors attribute the enhanced membrane affinity to fluorocarbon–hydrocarbon separation due to peptide aggregation in the lipid layer rather than to increased hydrophobicity.

Despite the incorporation of highly fluorinated amino acid residues into peptides by chemical synthesis on solid supports, fluorine-containing peptides and proteins are produced through expression from auxotrophic bacteria strains.[138] Addressing the issue of the nature of the "fluorine-effect" on protein stability and on hydrophobic domain interaction, prominent contributions have undoubtedly been made by the groups of Kumar and Tirrell. Their studies on α-helical coiled coil peptides containing highly fluorinated hydrophobic interaction surfaces will be discussed in Section VII.

Enkephalin Analogues

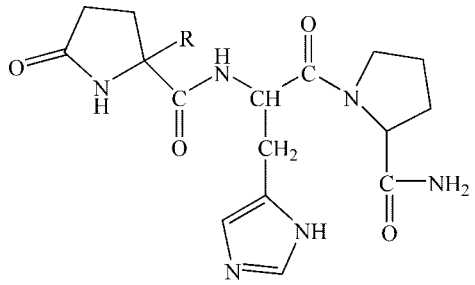
Remarkable results have been achieved in enhancing the activity of enkephalin analogues by incorporation of conformationally constrained fluorinated amino acids. The replacement of Gly^2 in Met-enkephalin with D-5,5,5-trifluoronorvaline by Ojima et al. increased the in vivo activity of the peptide by five orders of magnitude.[139] While the major part of the activity enhancement seems to be caused by an increased metabolic stability owing to the presence of the D-amino acid, about half an order of magnitude is contributed by a "fluorine effect". The enkephalin derivatives of Winkler et al. containing the fluoroalkylated amino acid 4,4-difluoro-2-aminobutyric acid showed a 100-fold higher receptor activity than that of the peptide containing the non-fluorinated analogue 2-aminobutyric acid (Scheme 11).[140] Here, this biological effect is clearly caused by specific favorable interactions between the receptor subsite and the fluoroalkyl group of the amino acid.



Scheme 11. Enkephalin analogues containing 2-aminobutyric acid (R = CH_3) or 4,4-difluoro-2-aminobutyric acid (R = CF_2H) in amino acid position 3.[140]

TRH Analogues

Thyreotropin releasing hormone (TRH: *p*Glu-His-Pro-NH_2) is the central stimulator of the secretion of thyroid stimulating hormone (TSH) by anterior pituitary cells, and it additionally stimulates the release of prolactin and growth hormone.[141] In vivo, the degradation of TRH is initiated by pyroglutamyl aminopeptidase II by selective cleavage of the *p*Glu-His bond.[142] According to a model study that was performed by our group, αTfm-substituted amino acids can increase the proteolytic stability of peptides by 100% when incorporated into the P_1 position (nomenclature according to Schechter and Berger[143]).[144] Therefore, pyroglutamic acid (*p*Glu) of TRH was substituted by (αTfm)*p*Glu (Scheme 12).[145]



Scheme 12. Structure of TRH; R = H: native peptide, R = Tfm: [(αTfm)*p*Glu[1]]-TRH.

As a result of the strong electron-withdrawing effect of the trifluoromethyl group, the substitution of *p*Glu within TRH by (α-Tfm)*p*Glu should, on the one hand, influence the structural conformation and the interaction between the peptide and the receptor, and, on the other, protect this hormone from hydrolysis by pyroglutamyl aminopeptidase II. The proteolytic stability of [(αTfm)*p*Glu[1]]-TRH against cleavage by membrane-bound TRH degrading ectoenzyme from Wistar rat anterior pituitary cells was determined by testing the inhibition rate of *p*Glu[^{3}H]-TRH degradation (Figure 3).[146]

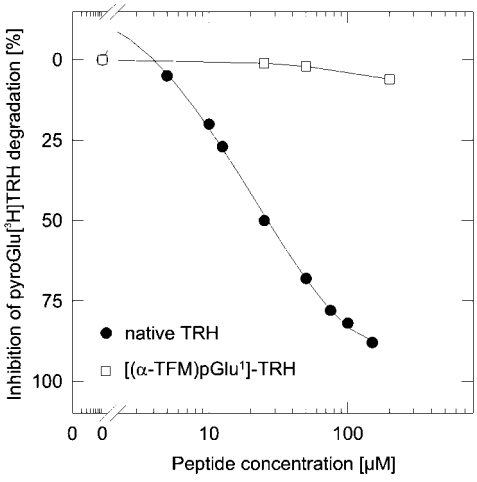


Figure 3. Determination of degradation stability of [(α-TFM) *p*Glu[1]]-TRH by *p*Glu[^{3}H]TRH degradation assay.

The results clearly show that [(αTfm)*p*Glu[1]]-TRH does not effectively inhibit the degradation of *p*Glu[^{3}H]-TRH. Thus, the αTfm substitution at position 1 in TRH results in complete resistance to proteolysis. However, αTfm-substituted TRH showed a binding affinity to the receptor that is 2–3 orders of magnitude lower than that of the native analogue. NMR spectroscopic characterization of both peptides suggests significant changes in the conformation of TRH.

The polarization effect of the Tfm group seems to decrease the capacity to form a hydrogen bond between the carboxy function of *p*Glu and the Pro amino group. This missing interaction might prevent a stable hairpin turn that is required for optimal binding to the receptor (Figure 4).

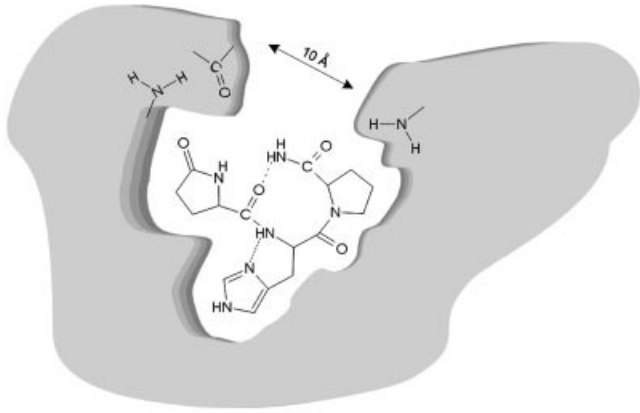


Figure 4. Model of the interaction between the TRH molecule and the receptor.

Analogues of Chemotactic Peptides

The impact of amino acid fluorine substitution on the activity of chemotactic peptides was studied systematically. A small library of the chemoattractant For-Met-Leu-Phe-NH_2 (fMLF) has been synthesized by substituting Leu in position 2 with different fluorinated amino acids that vary in the content of fluorine, the length of the fluorinated side chain, and the degree of alkylation at the α-carbon atom.[147] Chemoattractants, like fMLF that is produced by *Escherichia coli*,[148,149] are involved in the activation process of antibacterial chemotaxis, the directed movement of cells along a chemical gradient.[150] The response to infection starts with the movement of neutrophils toward the bacteria.[151] Although the cellular mechanism by which the neutrophils rapidly move to sites of infection remains a mystery, there is evidence that the chemotactic peptide fMLF interacts with a neutrophil receptor through binding to hydrophobic pockets.[152–154] Therefore, fMLF is a perfect system to study the hydrophobicity versus polarity, and the space filling of fluoroalkyl groups. The fluoromodified amino acids (*S*)- and (*R*)-$C^{\alpha,\alpha}$-difluoroethyl glycine (DfeGly), (*S*)- and (*R*)-$C^{\alpha,\alpha}$-trifluoromethyl alanine [(αTfm)-Ala], and (*S*)- and (*R*)-C^{α}-difluoromethyl alanine [(αDfm)-Ala] were incorporated into the middle position of the fMLF tripeptide. The αTfm and αDfm groups are known to induce considerable polarization effects on neighboring substituents. Due to the high electron density, the fluoroalkyl substituents afford peptide–receptor interactions that are unique.[144] In order to distinguish between electronic effects of the fluorine atoms and the steric demand of a $C^{\alpha,\alpha}$-dialkylation, we further studied the fMLF derivative For-Met-Aib-Phe-NH_2, which contains the $C^{\alpha,\alpha}$-tetrasubstituted nonfluorinated alanine analogue aminoisobutyric acid. The bioactivity of the peptide derivatives was studied with the use of a luminol-dependent chemiluminescence assay (Figure 5). Although the non-modified For-Met-Leu-Phe-NH_2 yielded the most intense response relative to all other tripeptides, the results clearly show that all of the new fMLF derivatives were active in generating reactive oxygen species. In general, the observed bioactivity was

dependent on both the nature of the substituent of the amino acid in position 2 and the absolute configuration of this building block. The most significant difference was observed between the two diastereomers of For-Met-DfeGly-Phe-NH_2. The (*S*)-DfeGly analogue, which has the fluorinated side chain in the same position as in native L-amino acids, showed more than twice the activity relative to the (*R*)-DfeGly peptide.

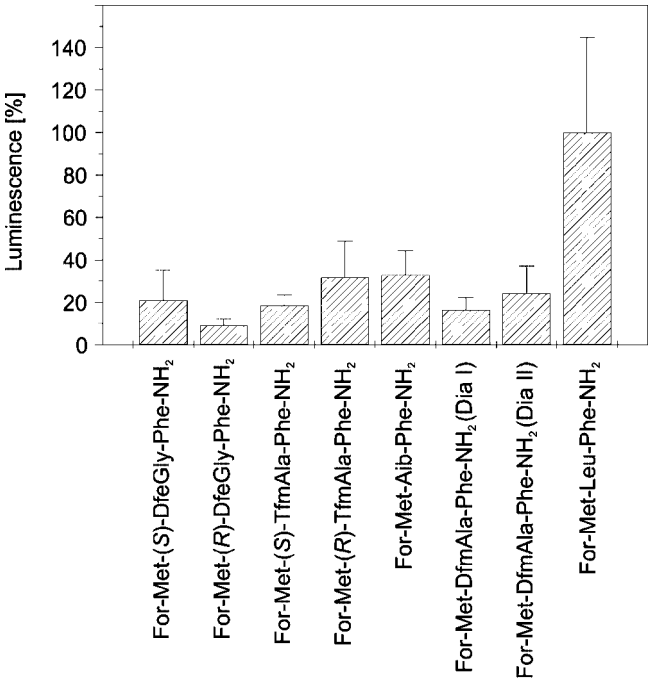


Figure 5. Integral values of the luminol chemiluminescence of the tripeptidestimulated cells. The integrals were determined over a time period of 10 min after stimulation.[147]

Obviously, the Leu^2-receptor binding pocket preferably interacts with amino acids that bear bulkier groups at the position of isobutyl in L-Leu. The peptide containing (*R*)-(αTfm)Ala, which is the diastereomer containing the trifluoromethyl group in the position of a native amino acid side chain, was more active in producing reactive oxygen species than its (*S*)-analogue. This proves an extended steric demand of the Tfm group. The absolute configuration of the two (αDfm)Ala-containing diastereomers has not been proven by crystal structure analysis yet. Therefore, these results cannot be interpreted with respect to space filling and polarity. Comparison of the bioactivities of the fluorinated fMLF derivatives with For-Met-Aib-Phe-NH_2 clearly shows that those of the analogues with the bulkier substituents in the C^α-position, which are comparable to side chains in L-amino acids, reached the same order of magnitude as that found for the Aib-substituted peptide. The bioactivity of the other diastereomers decreased. These findings indicate that the disturbing steric demand of C^α-substituents in the position that is comparable to side chains in D-amino acids has a higher impact on the binding capability to the receptor pocket than the extension in space filling of the L-amino acid side chain. The slightly lower activities observed for the (αDfm)Ala-substituted fMLF analogues than those for the For-Met-(αTfm)Ala-Phe-NH_2 derivatives indicate that the polar character of the Dfm group may weaken the ligand–receptor interaction within the hydrophobic receptor pocket.

VII. The Systematic Evaluation of the Interactions of Fluorinated Amino Acids Within a Native Polypeptide Environment

Studies on protease-catalyzed incorporation of different fluoroalkyl-substituted amino acids and on the impact of fluorinated building blocks on the structure, stability, and activity of biologically active peptides have imposingly shown the importance of the fluorine content and position of fluorination within an amino acid. However, we have also realized that a systematic evaluation of the impact of alkyl fluorination on steric demands as well as on polar and hydrophobic interactions is essential if we want to use fluorinated amino acids for the de novo design of peptides and proteins for the fine-tuning and the directed manipulation of peptide/protein interactions. Therefore, we have developed a screening system which enables the systematic investigation of fluorinated amino acids with respect to space filling, polarity, stability, and possible interaction partners. Those studies will contribute to a better understanding of how the characteristics of fluoroalkyl groups evolve in the context of a protein environment.

An essential requirement for the investigation of specific amino acid side-chain interactions and the influence of new building blocks on structural aspects of peptides and proteins is a model system that has the following characteristics:

The residue position to be substituted by the non-natural amino acid has to have well-determined amino acid residues defined as its interaction partners.

A stable secondary structure of this model system is required that tolerates the substitutions to be made.

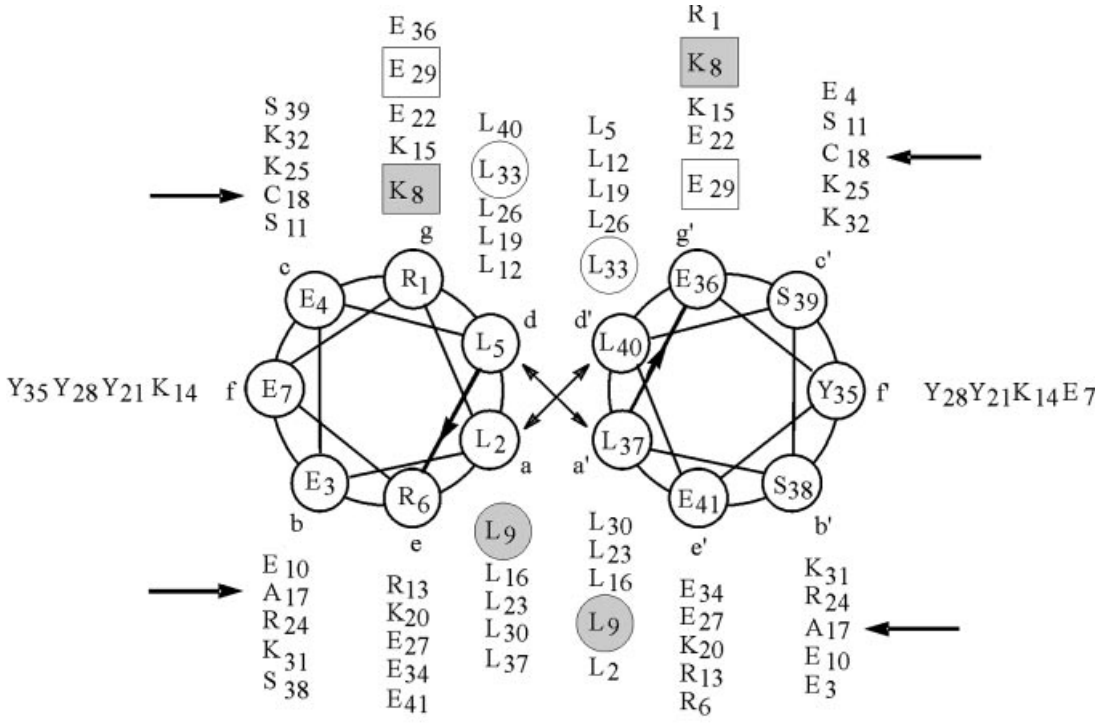
The model should allow a sensitive detection of the influence of the non-natural building block on peptide/protein conformation and stability.

α-Helical coiled coil peptides can perfectly serve as such model systems.[155,156] As they occur frequently in native proteins,[157–160] the structural principles of this motif have been analyzed in detail.[156,161–163] α-Helical coiled coils typically consist of 2–5 right-handed α-helices that are wrapped around each other to form a superhelical twist in a left-handed manner. The primary structure of each helix is characterized by a periodicity of seven residues, the so-called 4–3 heptad repeat that is commonly denoted as $(a\text{-}b\text{-}c\text{-}d\text{-}e\text{-}f\text{-}g)_n$. The positions a and d are typically occupied by nonpolar residues (Leu, Ile, Val, Met) and form a special interaction surface at the interface of the helices by hydrophobic core packing. In contrast, the positions e and g are exposed to the solvent and are often occupied by charged amino acids (the most common ones are Glu and Lys) forming interhelical ionic interactions. The hydrophobic core provides the major contribution to the structural sta-

bility of the coiled coil. In contrast, the interhelical ionic pairing positions e and g mainly dictate the orientation specificity (parallel versus antiparallel). Thus, the α-helical coiled coil provides two main domains that we can use to study fluorinated amino acids, a hydrophobic and a polar environment.

The Newly Developed Screening System Based on an α-Helical Coiled Coil Motif

The screening system is based on a homodimeric antiparallel coiled coil with a monomer length of 41 residues (Figure 6). The primary structure is designed for an antiparallel



peptide sequences:

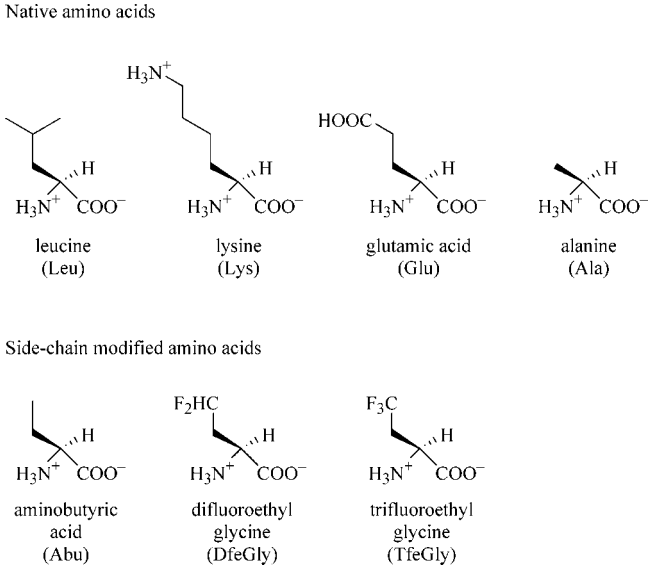
nucleophilic fragment	CLKYELRKLEYELKKLEYELSSLE
electrophilic fragments of native amino acids	
control sequence	Ac–RLEELREKLESLRKKLA
L9E	Ac–RLEELREK**E**ESLRKKLA
L9A	Ac–RLEELREK**A**ESLRKKLA
K8A	Ac–RLEELRE**A**LESLRKKLA
electrophilic fragments of side-chain modified amino acids	
L9Abu	Ac–RLEELREK**Abu**ESLRKKLA
K8Abu	Ac–RLEELRE**Abu**LESLRKKLA
L9DfeGly	Ac–RLEELREK**DfeGly**ESLRKKLA
K8DfeGly	Ac–RLEELRE**DfeGly**LESLRKKLA
L9TfeGly	Ac–RLEELREK**TfeGly**ESLRKKLA
K8TfeGly	Ac–RLEELRE**TfeGly**LESLRKKLA

Figure 6. Helical-wheel representation of the α-helical coiled coil peptides and their amino acid sequences. The substitution positions g,g′-K8 and a,a′-L9 (shaded in grey) and their interaction partners g′,g-E29 and d′,d-L33 (unshaded) are highlighted with squares (charged interface) and circles (hydrophobic interface). The ligation sites of the electrophilic and nucleophilic fragments are marked with arrows.

orientation of the helices through a maximum number of attractive interhelical ionic interactions of the residues in the e and g positions. In addition, a preference for antiparallel over parallel orientation[164] and dimerization over higher oligomers[165] is supported by a homogeneous hydrophobic core that is exclusively composed of Leu residues. The design concept, namely the antiparallel folding, of the α-helical coiled coil based screening system was proven by using high-resolution methods.[166]

The residues K8 in the g position within the charged domain and L9 in the a position within the hydrophobic core serve as substitution positions. The interaction partner of a fluorinated amino acid in position 8 is E29 and in position 9 is L33. The α-helical coiled coil structure motif is known to respond very sensitively to even a single variation within either of the recognition domains.[167] The detection of the influence of the fluoroalkyl amino acids on interhelical side chain–side chain interactions, and thus on coiled coil stability is accomplished by two simple screens. One screen measures the thermal stability of the peptide analogues by recording the unfolding profiles with CD spectroscopy. The second screen is based on the self-replicating property of α-helical coiled coil peptides (replicase reaction cycle).[168]

As discussed in Section I, a trifluoromethyl group is described to have space filling properties that are comparable to those of the isopropyl group. Accordingly, trifluoroethylglycine (TfeGly) should be as sterically demanding as native leucine within the lipophilic recognition domain of a coiled coil peptide. Therefore, our systematic study starts with fluorinated ethylglycines (Scheme 13).[169]



Scheme 13. Structures of all investigated amino acids within the substitution positions 8 and 9 of the electrophilic α-helical coiled coil fragments.

For comparative reasons we included variants containing alanine, ethylglycine (aminobutyric acid, Abu), difluoroethylglycine, and trifluoroethylglycine in the substitution position L9 of the hydrophobic core (E_{L9A}, E_{L9Abu}, $E_{L9DfeGly}$, and $E_{L9TfeGly}$), as well as E-variants with all of these amino acids in the position K8 of the charged domain (E_{K8A}, E_{K8Abu}, $E_{K8DfeGly}$, and $E_{K8TfeGly}$).

The Impact of Fluoroalkyl Substitution on Peptide/Protein Interactions

The variant E_{L9E} with a strong destabilizing Glu residue within the apolar core has been generated as an extreme example. Temperature-dependent CD spectroscopy in 5 M guanidine hydrochloride provided stability data in the form of melting curves for the ligation products (Figure 7). Thermal-denaturation experiments demonstrate the much stronger impact of substitutions within the hydrophobic core relative to those placed at the charged interface; this was as expected. The thermal stabilities of all variants differ from that of the control sequence, which proves that our screening system is sufficiently sensitive to detect even minor differences in side chains. This is best demonstrated with the fluorinated ethylglycine derivatives, which differ only in one single fluorine atom.

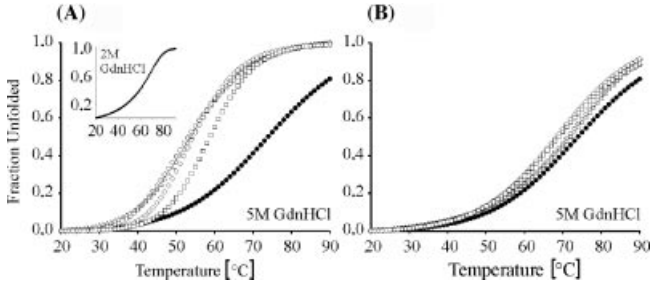


Figure 7. Thermal unfolding profiles (in 5 M guanidine hydrochloride) of α-helical coiled coil peptides of the control sequence (closed circles) and with substitutions in position 9 (A) and position 8 (B) with Ala (open circles), Abu (open triangles), DfeGly (open diamonds), and TfeGly (open squares). Inset: thermal unfolding profile of the L9E variant in 2 M guanidine hydrochloride, which was already fully unfolded at 20 °C in 5 M guanidine hydrochloride.

The L9E variant showed the strongest destabilization and did not form a dimer at room temperature in 5 M guanidine hydrochloride. All peptides modified in the hydrophobic domain were less stable than the control sequence, while L9A showed the strongest decrease in stability. Obviously, the short side chain of Ala creates a hole within the hydrophobic core, which is unfavorable for stable folding. Side-chain elongation by one methyl group (L9Abu) made only a minor contribution to stabilization, which is indicated by an increase in the melting point of less than 1 K (Table 3). In contrast, a further increase in side-chain volume and hydrophobicity by subsequent fluorination of the methyl group of Abu led to a stepwise increase in stability. An increase in melting point of 5 K was detected for L9TfeGly relative to the Abu-peptide. However, this secondary-structure stabilizing effect does not show a linear correlation with the order of fluorine substitution. The reason for this finding is the electron-withdrawing effect of the two fluorine substituents in the L9DfeGly variant, which results in an increased acidity of the proton attached.

The polarity of the CF_2H group was confirmed by a destabilization of the hydrophobic packing.

Table 3. Melting temperatures of the α-helical coiled coil peptides.

Peptide	T_m [°C][a]
Control sequence	73.9
L9Ala	53.2
L9Abu	54.0
L9DfeGly	54.4
L9TfeGly	59.0
K8Ala	71.3
K8Abu	71.9
K8DfeGly	68.3
K8TfeGly	68.9

[a] T_m is defined as the temperature at which 50% of the peptide is unfolded.

Considering the thermal unfolding data of L9-substituted coiled coils, the fluorination of a methyl group has a much higher impact on hydrophobic core stability than the elongation of the side chain by one methyl group. However, even the most stable variant of this series, L9TfeGly, showed a serious decrease in dimer stability relative to the control sequence. The opposite result was expected when considering that trifluoroethylglycine has a size similar to that of leucine and that fluoroalkyl groups have a high hydrophobicity (Figure 8).

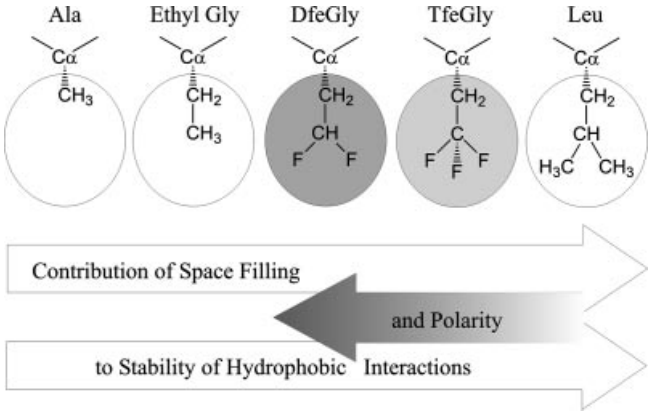


Figure 8. The impact of space filling properties and polarity of fluoroalkyl groups on the hydrophobic domain stability.

In fact, the groups of Tirrell and Kumar have demonstrated enhanced thermal as well as structural stabilities of parallel coiled coil peptides after incorporation of trifluoroleucines[170,171] and hexafluoroleucines[172] into the hydrophobic core of a coiled coil peptide. In these studies, the content of the fluoroalkylated amino acid correlated with the observed effect of stabilization.[173] Due to the parallel orientation of the peptides investigated by these authors, the fluorocarbon side chains generally interact with each other at the dimer interface. Interaction with native Leu residues is prevented, though. Therefore, less than 3% of heterodimeric assemblies were detected.[174,175] The stabilizing effect of the fluorination of the coiled coil core is obviously based on fluorine–fluorine interactions rather than on enhanced hydrophobicity. This led to the theory of "self-sorting" coiled coils.[174] In a recent publication, Bilgicer and Kumar discuss simultaneous hydrophobic and lipophobic fluorinated helical surfaces in coiled coil peptides.[176] They investigated the oligomerization behavior of peptides containing a coiled coil sequence composed of apolar residues within a membrane-like hydrophobic environment. The results showed that highly fluorinated dimerization interfaces are able to provide a driving force for the formation of oligomeric ensembles in lipid layers comparable to that provided by hydrogen bonding. This mediation of helix–helix interactions, consequently, originates from fluorocarbon–hydrocarbon phase separation, which supports the "fluorous-phase" explanation for coiled coil secondary structure stabilization. In addition, Arai et al. have shown that trifluoroethylglycines as hydrophobic residues in amphiphilic helices tend interact with each other.[177] In contrast to these studies, the group of Marsh carried out experiments with antiparallel tetrameric coiled coil peptides having the central two layers of the hydrophobic core repacked with 5,5,5,5′,5′,5′-hexafluoroleucine (hFLeu), and ascribed the observed increase in structural stability relative to the non-fluorinated analogue to the more hydrophobic nature of hFLeu rather than to specific fluorous interactions.[178] The greater hydrophobicity of hFLeu than of the canonical leucine has been quantified by partitioning experiments from *n*-heptanol into water. The resulting ΔΔG values were in good agreement with the additional stability each fluorinated leucine analogue had contributed to the modified peptide tetramer.

However, most of the studies suggested that fluorocarbon–fluorocarbon interactions are much stronger than both fluorocarbon–hydrocarbon and hydrocarbon–hydrocarbon interactions, a fact that has to be considered when fluoroalkylated amino acids are used for peptide and protein modification, e.g. in peptide-based drug design.

All substitutions within the charged coiled coil interaction domain showed a destabilization of the dimerization motif relative to that of the unmodified peptide, caused by an interruption of the K8–E29 salt bridge. While most attractive interhelical ion pairings in coiled coil peptides seem to stabilize the dimeric structure,[179,180] and the general rules for the optimization of intra- and interhelical salt bridges to achieve the optimum stability of the coiled coil have recently been drawn up,[181] examples of the reverse effect have been observed.[182] Both unfluorinated substituents K8A and K8Abu destabilized the coiled coil to a similar extent (lowering of the melting temperature by 2 K). The fluorination of ethylglycine led to a similar destabilization (ca. 3 K). This effect is, obviously, due to an enlargement of the hydrophobic side chains, which are exposed to the polar solvent. As observed in the case of the hydrophobic recognition domain, the fluorination of a methyl group had a higher impact on the lipophilic character of an amino acid side chain than the addition of one methyl group.

The replicase reaction cycle of the second screen is a template-assisted auto-catalyzed self-replication process. The efficiency of template-assisted peptide bond formation strongly depends on the extent to which the interacting

amino acid side chains of both recognition domains complement each other. The fluorine substitutions have an impact on both the association with the template and the dissociation of the coiled coil homodimer formed. Thus, the replicase reaction rates are affected by the non-natural amino acids. As a result, they give detailed information on the influence of the fluoroalkyl substituent on association and dissociation processes and on the strength of the side chain–side chain interactions within hydrophobic and charged peptide interfaces. Our ongoing investigations on these processes will be presented at a later date.

In summary, the screening system described herein has proven to be a valuable and sensitive tool for the investigation of the electronic and space filling properties of fluorocarbon residues within a native polypeptide environment. Depending on the polarity of the immediate substitution environment, the impact of fluoroalkylation on the thermodynamic and kinetic aspects of peptide and protein folding can be studied. Furthermore, it allows a systematic evaluation of fluorinated amino acids that differ in side-chain length and fluorine content for peptide design and protein engineering.

This review gives some insight into the unique role of fluorine in medicinal chemistry and protein analysis with respect to its considerable potency for application in peptide and protein engineering. In this context, we have given an introduction to the work of our research group, which focuses on varied topics such as the synthesis of fluorinated amino acids, the development of efficient strategies for their incorporation into peptides and proteins, and the impact of these amino acids on pharmacological parameters of bioactive peptides. The effects that we have discussed with respect to the interaction of fluorinated amino acids with the active site of several proteases and amidases provide a wealth of solid information on how fluoroalkyl groups interact with enzymes, how they might interact with receptors, and how these groups change the interaction profile of a natural peptide. Furthermore, we have described a screening system that was developed for a systematic evaluation of the physicochemical properties of fluoroalkyl-substituted amino acids and their interaction with native polypeptides. This screening system gives new, valuable information that may extend the use of fluorine to a rational design of peptide-based drug molecules in the future.

Acknowledgments

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