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Highlight

Biosynthetic Incorporation of Fluorinated Amino Acids into Peptides and Proteins

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Native and engineered protein biosynthetic machinery processes a wide range of fluorinated α-amino acids for incorporation into peptides and proteins, either as substitutes for structurally similar amino acids normally found in proteins, or as additional ones. In the former case, replacement occurs wherever the normal amino acid is encoded, while the latter method is site-specific. The fluorinated peptides have a diverse variety of interesting properties. The biochemical synthetic methods are straightforward, to the point that they should routinely be assessed as alternatives to traditional solid- and solution-phase peptide synthesis.

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Introduction

It is generally understood that ribosome-derived peptides and proteins are comprised of twenty α-amino acids and, indeed, life depends on the fidelity of their incorporation. Nevertheless, many others are also incorporated. Usually this only occurs when those other amino acids are structurally similar to, and compete for incorporation with, one normally found in proteins and in this regard amino acids having fluorine in place of hydrogen often satisfy this criterion. Although the earlier misconception that fluorine is similar in size to hydrogen has now been largely dispelled,[1] often the fluorine–hydrogen substitution has negligible impact on the binding of compounds to enzymes and other proteins[2] including those involved in peptide biosynthesis. Accordingly, peptides and proteins containing fluorinated amino acids can be prepared using natural protein biosynthetic machinery as an alternative to more conventional solid- and solution-phase peptide synthesis techniques. Through bioengineering the scope of this method has been greatly extended. This article provides an overview of those fluorinated amino acids that are incorporated, biochemical methods of peptide and protein synthesis that favour this, and properties of the fluorinated products.

Incorporation in Natural Systems

Fluorinated compounds are very rare in nature and, to the best of our knowledge, (2*S*,3*S*)-4-fluorothreonine (**1**) (Fig. 1) which has been isolated from *Streptomyces cattleya* is the only α-amino acid in this group.[3] Naturally occurring fluorinated peptides and proteins are unknown. Even so, numerous methods have been developed for the synthesis of a wide range of fluorinated amino acids,[4,5] and the incorporation of many of these has been

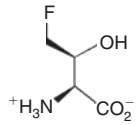

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Fig. 1. The only known naturally occurring fluorinated α-amino acid.

investigated (Figs 2–4). Those substituted with fluorine at either the α- or β-position are relatively difficult to prepare due to their propensity to undergo dehydrofluorination and, even though methods have been developed for chemical synthesis of corresponding peptides,[6,7] these amino acids are unsuitable for biochemical approaches.

Fluoroaromatic amino acids are arguably the easiest to prepare and the most stable (many of these are now commercially available), which is probably one reason why 4-fluorophenylalanine (**2a**) (Fig. 2) was the first to be studied as a substitute for a normal amino acid in its biosynthetic incorporation into proteins in natural systems. In the early reports the evidence of incorporation was only indirect. Thus, the fluoride **2a** was first investigated as a replacement for (*S*)-phenylalanine (Phe) by monitoring its effects on the growth of *Lactobacillus arabinosus*.[8] Direct analytical methods and ^{19}F NMR spectroscopy in particular were used later, to establish, for example, that feeding rabbits with the fluoride **2a** resulted in the production of carbonic anhydrase in which each of the Phe residues was partially (~5%) replaced,[9] and that egg white lysozyme isolated from birds fed with the 4-, 3-, and 2-fluorophenyalanines (**2a**–**c**) and the 4-, 5-, and 6-fluorotryptophans (**3a**–**c**) partly incorporated those amino acids in place of each Phe and (*S*)-tryptophan, respectively.[10]

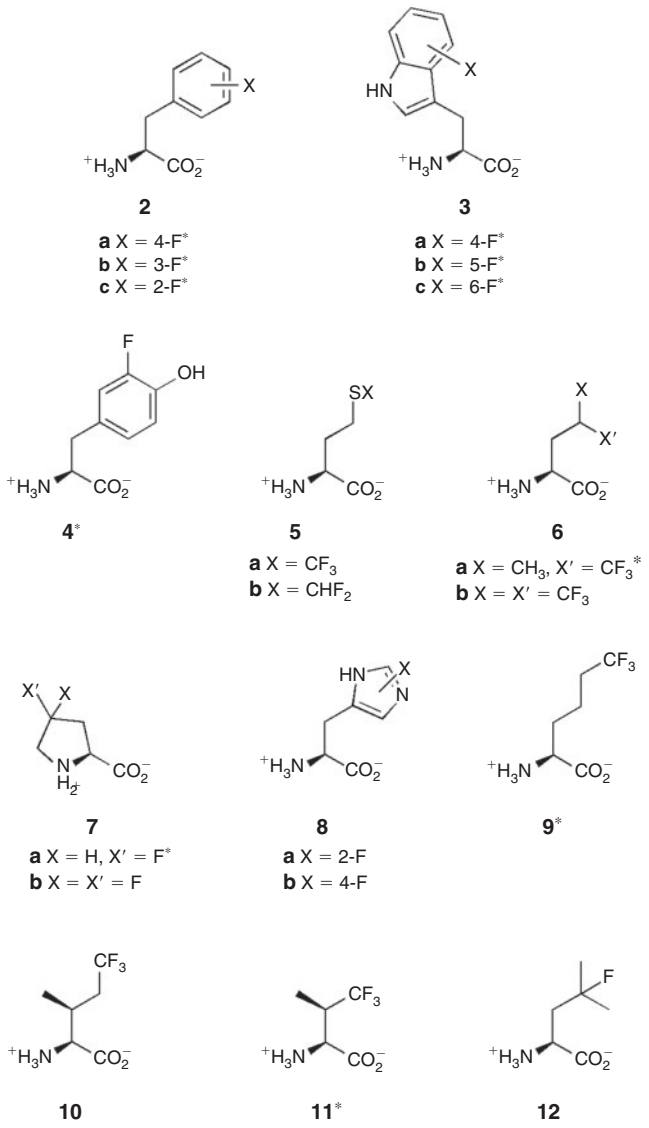

Fig. 2. Fluorinated α-amino acids that compete for incorporation with those amino acids normally found in proteins. *Denotes compound is commercially available.

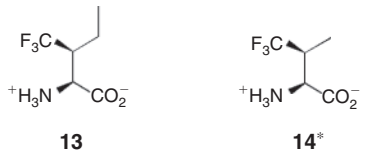

Fig. 3. Fluorinated amino acids that do not compete for incorporation. *Denotes compound is commercially available.

Use of Amino Acid Auxotrophic Bacteria

For practical peptide and protein synthesis, higher yields of materials that are more easily isolated and purified are obtained through overexpression in bacteria. When auxotrophic strains that lack the ability to synthesise one or more of the amino acids normally found in proteins are used for this purpose, there is the added advantage of much greater levels of incorporation of other structurally similar amino acids due to reduced competition. Accordingly, (*S*)-tyrosine (Tyr), (*S*)-methionine (Met), (*S*)-leucine (Leu), (*S*)-proline (Pro), and (*S*)-histidine *Escherichia coli* auxotrophs have been used to produce peptides containing high levels of the fluorinated amino acids **4**,[11] **5a**,[12] **5b**,[13] (2*S*,4*S*)- and (2*S*,4*R*)-**6a**,[14] (2*S*,4*S*)- and (2*S*,4*R*)-**7a**,[15,16] and **8a**, **b**,[17] respectively.

For ribosomal peptide synthesis, amino acids are first activated by corresponding aminoacyl-tRNA synthetases for attachment to their cognate tRNAs. Whether or not fluorinated amino acids are incorporated depends directly on the extent of their activation and several strategies have been developed to increase this. One is to exploit mutant synthetases that have been identified through screening as having greater activity for the fluorides. This has been demonstrated through the use of an *Escherichia coli* strain that co-expresses a Met-tRNA synthetase variant, to facilitate protein synthesis with the trifluoronorleucine **9** as a Met replacement.[18] This substitution does not occur in the absence of the unusual synthetase. Another approach is to use bacterial hosts engineered to produce enhanced levels of wild-type synthetase, as illustrated with hexafluoroleucine **6b**,[19] trifluoroisoleucine **10**,[20] and difluoroproline **7b**.[21] These were only activated efficiently enough to support protein synthesis in systems co-expressing Leu-, (2*S*,3*S*)-isoleucine- (Ile-), and Pro-tRNA synthetase, respectively. Even with additional Leu-tRNA synthetase, the level of activation of the trifluoroisoleucine **13** (Fig. 3) remained insufficient.

(2*S*,3*R*)-4,4,4-Trifluorovaline (**11**) does not substitute for (*S*)-valine (Val) during protein synthesis with conventional *Escherichia coli* auxotrophs. In an unusual twist on the strategy of addressing this with enhanced levels of synthetase, through co-expression of Val-tRNA synthetase the fluoride **11** replaces Val, but with additional Ile-tRNA synthetase it substitutes for Ile.[22] That is, the synthetase determines which of the two normal amino acids is replaced. (2*S*,3*S*)-4,4,4-Trifluorovaline (**14**) does not incorporate instead of either Val or Ile, even with co-expression of either Val- or Ile-tRNA synthetase. This shows that not all those analogues of the amino acids normally found in proteins having fluorine in place of hydrogen are suitable for biochemical protein synthesis, yet the examples of the fluorides **9** and **11** replacing Met and Ile, respectively, demonstrate that the method is not limited to that substitution. In the former case, fluoride **9** differs from Met by having a CH_2CF_3 group instead of SCH_3 and, in the latter, in place of CH_2CH_3 there is CF_3.

Use of Cell-Free Peptide and Protein Synthesis

More recently, cell-free expression has emerged as a useful tool for peptide and protein synthesis. By comparison with cellular expression systems, the cell-free approach involves the prior extraction and partial purification of cellular material, which is then supplemented with the necessary DNA, amino acids, and other ingredients to enable the synthesis. The cell-free expression systems can be biased towards incorporation of amino acids normally not found in proteins by withholding the normal amino acids with which they compete,[23,24] such that fluorinated amino acids processed through cellular expression are also taken up by the cell-free method, often to greater extents. Cellular extracts used for cell-free expression also contain enzymes that catalyse the removal of a wide range of the protecting groups employed in the synthesis of amino acids, so the deprotection of an amino acid to be incorporated can be carried out in situ to avoid separate processing steps.[25,26] This is particularly useful with unstable amino acids, such as fluoroleucine **12**. It rapidly decomposes through lactone formation but this can be avoided

by generating it in situ from the corresponding amino acid hydrazide, whereupon it substitutes for Leu in peptides and proteins.

Site-Specific Incorporation

When a fluoride competes for incorporation with an amino acid normally found in proteins, as is the case in the examples discussed above, substitution occurs whenever the mRNA encodes the normal amino acid. This is said to be residue-specific. Alternatively, various molecular engineering strategies such as supplementation of cell-free systems with an appropriate mutant aminoacyl-tRNA synthetase and cognate suppressor tRNA allow incorporation of an unnatural amino acid in addition to the normal ones, at a single site or in a site-specific manner.[27,28] Fluoro amino acids **15**–**23** that have been introduced into proteins in this manner are illustrated in Fig. 4.[29–36] An added advantage of this method is that the unnatural amino acid does not need to structurally resemble any normal one. Indeed, it should be dissimilar so as to avoid any background residue-specific incorporation. In this regard, the nitrobenzyltyrosines **23a**–**c** were developed as photolabile, masked fluorotyrosines in order to accomplish site-specific incorporation while preventing substitution of other Tyr residues.[36] A complementary approach for site-specific incorporation in large peptides and proteins is to prepare a fragment peptide comprising the unnatural amino acid through conventional solid- or solution-phase synthesis, then use chemical ligation to fuse this to the remainder of the target produced biosynthetically.[37]

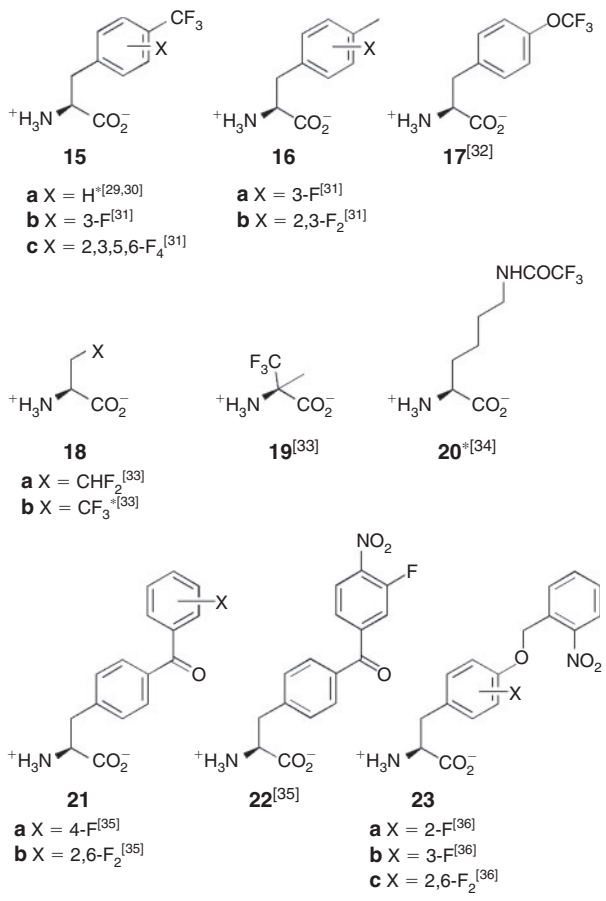

Fig. 4. Fluorinated α-amino acids that have been incorporated site-specifically. *Denotes compound is commercially available.

Applications

A major impetus for producing peptides and proteins comprising fluorinated amino acids is their utility in ^{19}F NMR spectroscopy, for determining structures and probing conformational changes and ligand binding,[38,39] and many of the examples discussed above were developed for this purpose. The introduction of fluorine also increases the resistance of peptides to thermal and chemical denaturation,[20,40–43] oxidation,[44] and proteolysis.[45,46] Fluorinated amino acids can also be used to manipulate hydrophobicity[47] and local electronic environments,[36,48] as well as alter the reactivity of catalytic groups.[49,50] For example, the kinetics of an isomerase enzyme have been modified by replacing the active site, catalytic tyrosine with fluorinated analogues having altered acidities.[50] Fluorine (^{18}F) is used as a tracer in positron emission tomography (PET) and amino acids labelled with fluorine have been employed to monitor protein synthesis by this method.[51] Incorporation of fluorinated amino acids also offers possibilities for the redesign of ribosomally-derived antibiotics[52,53] and hormones[54,55] having modified activities. Further information on the uses and applications of peptides prepared from fluorinated amino acids is provided in recent articles by Merkel and Budisa[56] and Marsh.[57]

Conclusions

It follows that there is very broad scope to use native and engineered protein biosynthetic machinery to incorporate a wide range of fluorinated amino acids into peptides, for diverse applications. This approach is not without limitations. Consequently, traditional solid- and solution-phase peptide synthesis is likely to remain the method of choice for synthesis of short peptides, particularly those containing backbone-modified amino acids, but for larger sequences biochemical methods are much more convenient, because they are efficient, scalable, and provide complete control over amino acid sequence. The biosynthetic reagents are now commercially available or easily prepared, and their use is straightforward, to the extent that the viability of using them in any planned synthesis of a peptide is worthy of consideration.

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