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Biosynthesis and stability of coiled-coil peptides containing (2*S*,4*R*)-5,5,5-trifluoroleucine and (2*S*,4*S*)-5,5,5-trifluoroleucine

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Introduction of non-canonical amino acids into proteins is a powerful method for the creation of macromolecules with novel properties.[1–3] In particular, 5,5,5-trifluoroleucine (TFL, **2**) has been utilized as a more hydrophobic surrogate of leucine in various contexts (Figure 1).[4, 5] When incorporated into the hydrophobic cores of certain coiled-coil proteins, TFL triggers an increase in stability, rendering proteins more resistant to thermal and chemical denaturation.[6, 7] Furthermore, despite the larger volume of CF_3 compared to CH_3, protein structure and activity can be retained upon replacement of leucine (Leu, **1**) by TFL.[8, 9]

Leu contains one stereocenter (C_α), which has the *S*-configuration. Replacement of a methyl group by a trifluoromethyl group at C_γ introduces an additional stereocenter and yields the two diastereoisomers (2*S*,4*S*)-5,5,5-trifluoroleucine (**3**) and (2*S*,4*R*)-5,5,5-trifluoroleucine (**4**) (Figure 1). Here we report the effects of TFL stereochemistry on coiled-coil peptide biosynthesis and stability. We demonstrate that both **3** and **4** are activated and incorporated into recombinant peptides prepared in *Escherichia coli.* Coiled-coil homodimers of peptides bearing **3** or **4** exhibit increased stability when compared to dimers of the Leu form of the peptide. An equimolar mixture of the two fluorinated peptides forms a heterodimer of modestly enhanced thermal stability relative to the homodimers.

The fidelity of translation is governed in large part by the activation of amino acids by their cognate aminoacyl-tRNA synthetases (AARS).[10] Although some AARSs are known to tolerate non-canonical substrates, amino acid activation can be acutely sensitive to side-chain stereochemistry. For example, isoleucine contains two stereocenters, both of *S*-

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configuration: one at C_α and another at C_β. The (2*S*,3*R*)-isomer (*allo*-isoleucine) is not incorporated into proteins, although there is evidence that it is bound and activated by isoleucyl-tRNA synthetase (IleRS).[11–14] Stereochemistry can also determine the fate of non-canonical amino acids as possible substrates for protein synthesis in *E. coli*. For instance, the isoleucine analogue, 2-amino-3-methyl-4-pentenoic acid, is accepted only in its (2*S*,3*S*)-form, while the valine (or isoleucine) analogue, 4,4,4-trifluorovaline (TFV), is active only in its (2*S*,3*R*)-form.[15, 16] We sought to explore whether leucyl-tRNA synthetase (LeuRS) exhibits a stereochemical preference with respect to activation of TFL.

Coiled-coil peptides constitute simple model systems for use in investigations of protein biosynthesis and stability.[17–20] Stereoisomers **3** and **4** were prepared (Scheme 1S, supporting information) and evaluated for incorporation into coiled-coil peptide A1[18] (Figure 1) in an *E. coli* strain auxotrophic for leucine. A1 was also expressed in media supplemented with **1** or **2**. Following purification on Ni–nitrilotriacetic acid affinity columns, protein yields were determined to be 18 ± 4 mg/L and 9 ± 3 mg/L upon incubation with **3** and **4**, respectively, compared to 45 ± 6 mg/L for A1 prepared with **1**. Peptides containing **3** and **4** were designated SS-A1 and SR-A1, respectively.

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry analysis of A1 fragments obtained by trypsin digestion was performed to assess the extent of substitution by **3** or **4**. Fragment **L**KNEIED**L**KAEIGD**L**NNTSGIR, corresponding to residues 46–67 in A1, contains three leucine residues (shown in bold type). Fragments that correspond to replacement at 0, 1, 2, and 3 sites by either **3** or **4** were observed (Figure 2). The expected mass increment of 54 Da was visible for each leucine residue replaced by TFL. Incomplete replacement of **1** most likely reflects a persistent pool of the natural amino acid replenished by cellular protein turnover.[19] The distribution of peak intensities (though not simply related to the relative abundances of fragments) is roughly consistent with unbiased substitution of **3** or **4**, and suggests a slight preference for incorporation of **3** (90% replacement of Leu in SS-A1) versus **4** (82% in SR-A1). Quantitative amino acid analysis was consistent with the MALDI results, showing 91% replacement in SS-A1 and 80% in SR-A1.

The relative rates of activation of **1** and fluorinated analogues **2–4** by LeuRS were determined by an *in vitro* ATP-PP_i exchange assay. The kinetic parameters are shown in Table 1. The relative k_{cat}/K_m values show that **3** is a slightly better substrate than **4**, consistent with the modest differences in yield and incorporation levels described above. As expected, the apparent k_{cat}/K_m for **2** fell between the values for **3** and **4** (Table 1). The activation rates for both **3** and **4** are within the range of rates that have been shown to support protein synthesis in conventional hosts cultured with other non-canonical amino acids.[21] Although a slight stereochemical preference is observed with respect to activation of **3** vs. **4**, this result stands in sharp contrast to the absolute selectivity imposed by IleRS and ValRS in the activation of TFV, in which only the *2S,3R* isomer is tolerated.[15, 16] Furthermore, the fact that both **3** and **4** can be incorporated into proteins in *E. coli* is consistent with previous work showing that hexafluoroleucine is activated by LeuRS.[21] All of these results indicate that fluorination at either of the C_δ positions is tolerated by LeuRS.

The secondary structures of all four A1 peptides were examined by circular dichroism (CD) spectroscopy. Because the peptides can form dimers, Leu-A1, SR-A1 and SS-A1 represent homodimers whereas the equimolar mixture of SR-A1 and SS-A1 can form either homo- or heterodimers. All four spectra were nearly identical at 1°C, and indicated ca. 90% helical content in each peptide as judged from the molar ellipticity at 222 nm (Figure 3A).[22, 23] CD provided no evidence that fluorination affects the secondary structure of A1.

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Previous studies have shown that incorporation of fluorinated amino acids into coiled-coil peptides and α-helical bundles results in enhanced stability;[7, 8, 19, 20, 24] the extent of stabilization varies depending on the identity of the fluorinated analogue.[19, 24] To determine whether the stereochemistry of TFL affects the extent of stabilization of A1, thermal denaturation was monitored by CD spectroscopy (Figure 3B). For both SR-A1 and SS-A1, the thermal melting temperature (T_m) was 65°C, 11°C higher than that of Leu-A1 (T_m = 55°C). The equimolar mixture of SR-A1 and SS-A1 exhibited a T_m of 68°C. This additional 3°C increase in T_m, suggested that SR-A1 and SS-A1 form heterodimers rather than a mixture of homodimers (Table 1S, supporting information). When a mixture of the (2*S*,4*S*)- and (2*S*,4*R*)-forms of TFL was used for expression of A1, ΔT_m was 13 °C,[6] nearly identical to that observed for equimolar mixtures of SS-A1 and RR-A1.

The results reported here demonstrate that both the SR- and SS-isomers of TFL are incorporated into proteins expressed in *E. coli*; the S- isomer is activated at a slightly higher rate by LeuRS. The higher activation rate leads to higher protein yields for SS-A1 relative to SR-A1 and to increased levels of incorporation of the fluorinated analogue. Neither stereoisomer appears to alter the coiled-coil structure of A1. Replacement of Leu by either isomer enhances the thermostability of A1; the heterodimer of SS- and SR-A1 shows an additional modest increase in stability. Experiments are underway to extend these findings and explore more fully the influence of side-chain fluorination on protein stability.

Experimental Section

Synthesis of 3 and 4

Amino acids **3** and **4** were prepared as described previously (see supporting information).[25–27]

Protein biosynthesis and purification

Leucine auxotrophic strain LAM1000 transformed with pREP4 (Qiagen) was used as the *E. coli* host to express A1, which was encoded within pQEA1 under the control of a *lac* promoter. Protein expression and purification were performed as described previously.[7] Protein concentrations were determined via UV spectroscopy.

Protein characterization

CD data were collected on an Aviv 62DS spectropolarimeter (Lakewood, NJ) using a 1 mm pathlength cell. Wavelength scans were taken from 195 to 250 nm, with points taken every 1 nm. Temperature scans were performed from 0–95°C in 1.5°C steps. Each plot represents an average of 3 scans.

Activation kinetics

An N-terminal His_6-LeuRS fusion was expressed and purified as previously reported.[21] Measurement of the rates of activation of leucine and analogues was performed by an ATP-PP_i exchange assay. The assay buffer conditions were 30 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 2 mM ATP and 2 mM [^{32}P]-PP_i (0.5 TBq/mol). A fixed concentration of 75 nM of His_6-LeuRS was used. Depending upon the activity of the enzyme toward the substrate, the following substrate concentrations ranges were used (**1**: 0.6–312.5 μM; **2**, **3**, **4**: 6.1–6250 μM). Once the reaction was completed, the reaction mixture was quenched by addition of 200 mM PP_i, 7% w/v $HClO_4$ and 3% activated charcoal. The charcoal was washed twice and measured on a scintillation counter. Kinetic data were fit using non-linear regression analysis.

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Supplementary Material

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Acknowledgments

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MRGSHHHHHHGSMA SGD**L**ENE VAQ**L**ERE VRS**L**EDE

a b c d e f g a b c d e f g a b c d e f g a b c d e f g
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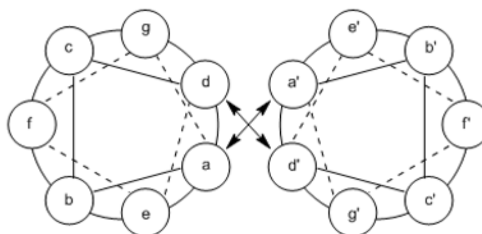

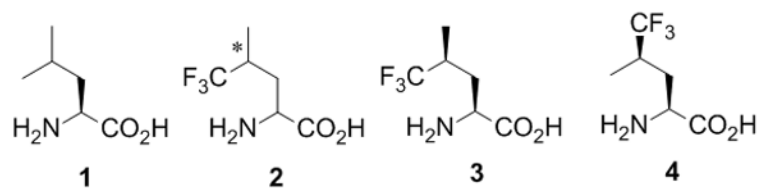

Figure 1.
A1 peptide sequence and helical wheel diagram of the dimer in which leucines are highlighted in bold. Structures of leucine (**1**) and the trifluoromethyl analogs (**2–4**). The asterisk in structure **2** denotes unresolved stereochemistry at the 4-position.

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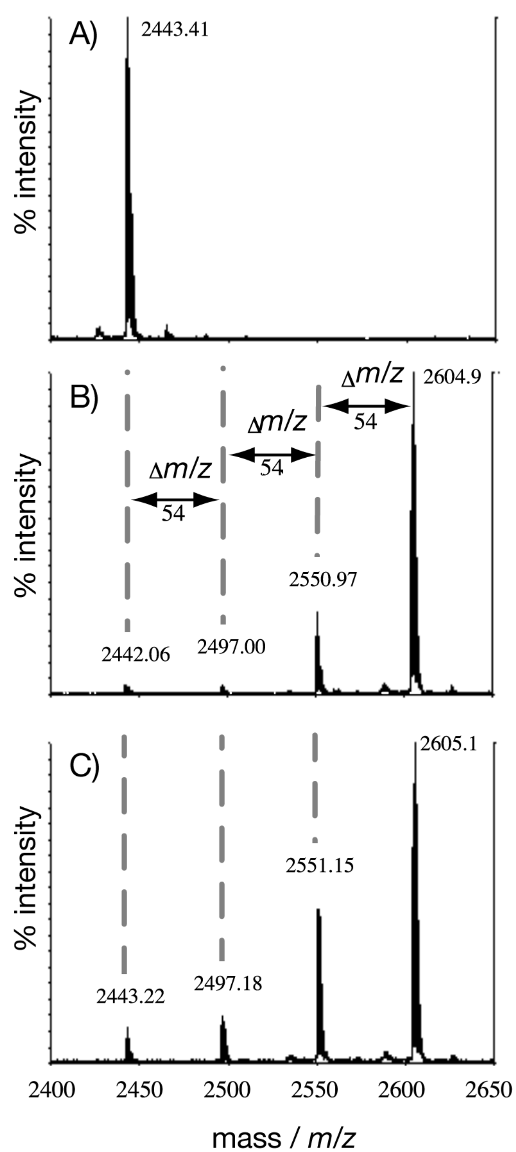

Figure 2.

MALDI mass spectra of a tryptic fragment of A1 (residues 46–67) containing 3 leucine positions. A1 was expressed in media supplemented with either **1** (A), **3** (B), or **4** (C). Fragments corresponding to 0, 1, 2, and 3 sites of substitution are represented as ~2442, 2497, 2551 and 2605 Da, respectively.

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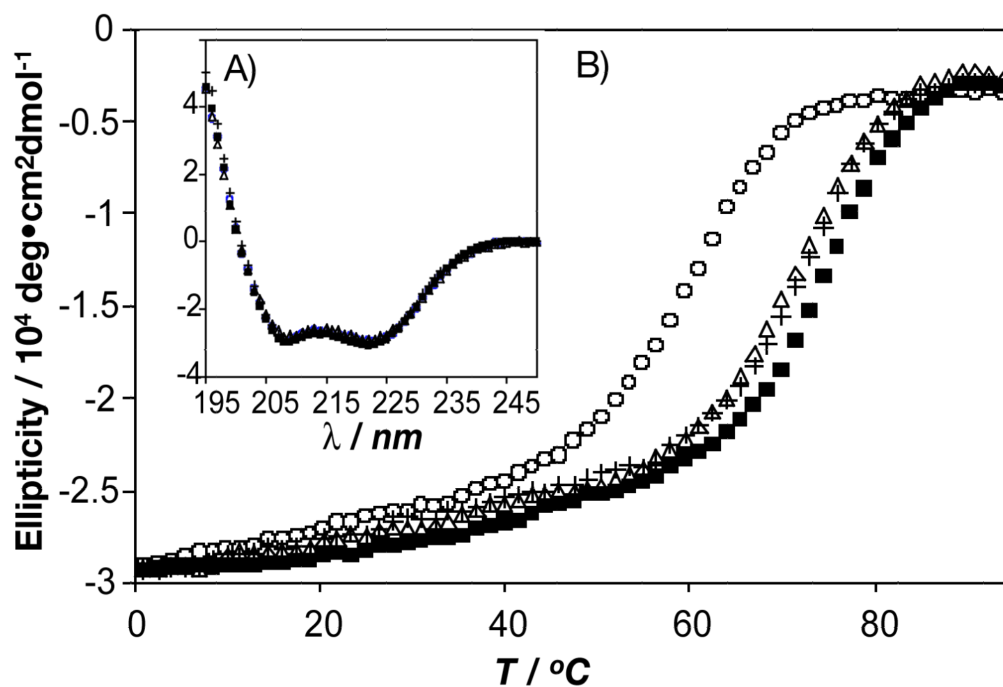

Figure 3.
CD spectra of Leu-A1 (○), SR-A1 (△), SS-A1 (+), and an equimolar mixture of SR-A1 and SS-A1 (■). A) Wavelength scan at 1 °C, 10 μM protein concentration, PBS buffer, pH 7.4. B) Thermal denaturation (1.5 °C interval, 1 minute equilibration time, 10 second averaging time) at 10 μM protein concentration, PBS buffer, pH 7.4.

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Table 1

Kinetic parameters for activation of **1 – 4** by *E. coli* LeuRS[a]

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (*rel*)
1	16.9 ± 4.5	4.22 ± 0.35	1
2	659 ± 103	0.40 ± 0.03	1/412
3	252 ± 92	0.59 ± 0.05	1/107
4	708 ± 280	0.19 ± 0.02	1/933

[a]Substrate **1** was used as the L-isomer; **2** as a mixture of (2*S*,4*S*), (2*S*,4*R*), (2*R*,4*S*) and (2*R*,4*R*) forms; **3** as the (2*S*,4*S*) form and **4** as the (2*S*, 4*R*) form.