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Chemoenzymatic Synthesis of (*S*)-Hexafluoroleucine and (*S*)-Tetrafluoroleucine

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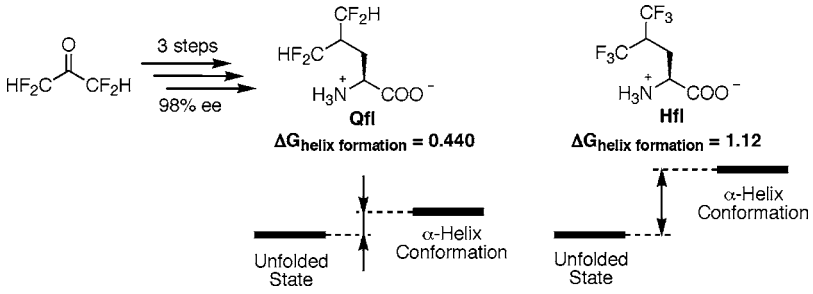
ABSTRACT



We have developed a short chemoenzymatic synthesis for both (*S*)-5,5,5,5′,5′,5′-hexafluoroleucine (Hfl) and (*S*)-5,5,5,5′,5′-tetrafluoroleucine (Qfl) on gram scale. Qfl was incorporated into a peptide using standard solid-phase peptide synthesis protocols to measure its helix propensity. The helix propensity for Qfl is 0.68 kcal·mol^{-1} more favorable compared to Hfl.

Highly fluorinated amino acids have been used to stabilize proteins[1−10] for potential applications in industrial scale biotransformations, biosensors, and protein therapeutics.[11] In particular, leucine (Leu) has been replaced with either diastereomeric mixtures of (2*S*)-5,5,5-trifluoroleucine (Tfl)[1,2,5] or enantiomerically pure (*S*)-5,5,5,5′,5′,5′-hexafluoroleucine (Hfl)[3,6,8,9] to stabilize helical proteins (Figure 1). Despite the various published procedures for synthesizing Hfl,[3,12−14] the generation of enantiomerically pure Hfl remains challenging. Furthermore, we recently showed that the helix propensity decreases drastically (>1 kcal·mol^{-1}) upon replacing Leu

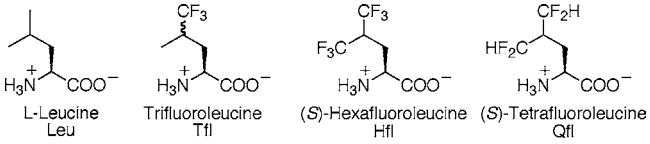


Figure 1. Leucine and fluorinated leucine analogues.

(1) Tang, Y.; Ghirlanda, G.; Vaidehi, N.; Kua, J.; Mainz, D. T.; Goddard, W. A., III; DeGrado, W. F.; Tirrell, D. A. *Biochemistry* **2001**, *40*, 2790−2796.
(2) Tang, Y.; Ghirlanda, G.; Petka, W. A.; Nakajima, T.; DeGrado, W. F.; Tirrell, D. A. *Angew. Chem., Int. Ed.* **2001**, *40*, 1494−1496.
(3) Tang, Y.; Tirrell, D. A. *J. Am. Chem. Soc.* **2001**, *123*, 11089−11090.
(4) Son, S.; Tanrikulu, I. C.; Tirrell, D. A. *ChemBioChem* **2006**, *7*, 1251−1257.
(5) Bilgiçer, B.; Fichera, A.; Kumar, K. *J. Am. Chem. Soc.* **2001**, *123*, 4393−4399.
(6) Bilgiçer, B.; Kumar, K. *Tetrahedron* **2002**, *58*, 4105−4112.
(7) Horng, J.-C.; Raleigh, D. P. *J. Am. Chem. Soc.* **2003**, *125*, 9286−9287.
(8) Lee, K.-H.; Lee, H.-Y.; Slutsky, M. M.; Anderson, J. T.; Marsh, E. N. G. *Biochemistry* **2004**, *43*, 16277−16284.
(9) Lee, H.-Y.; Lee, K.-H.; Al-Hashimi, H. M.; Marsh, E. N. G. *J. Am. Chem. Soc.* **2006**, *128*, 337−343.
(10) Woll, M. G.; Hadley, E. B.; Mecozzi, S.; Gellman, S. H. *J. Am. Chem. Soc.* **2006**, *128*, 15932−15933.
(11) Ritter, S. K. *Chem. Eng. News* **2007**, *85*(37), 36−37.
(12) Zhang, C.; Ludin, C.; Eberle, M. K.; Stoeckli-Evans, H.; Keese, R. *Helv. Chim. Acta* **1998**, *81*, 174−181.
(13) Xing, X.; Fichera, A.; Kumar, K. *Org. Lett.* **2001**, *3*, 1285−1286.
(14) Anderson, J. T.; Toogood, P. L.; Marsh, E. N. G. *Org. Lett.* **2002**, *4*, 4281−4283.

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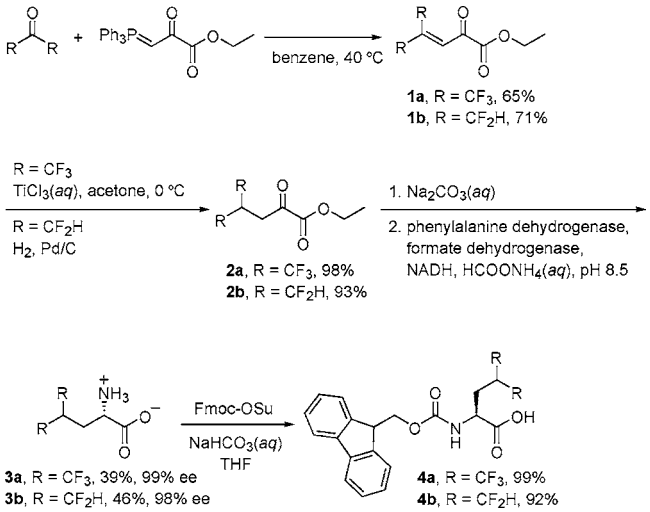
with Hfl,[15] suggesting the need to develop novel fluoro-amino acids with more favorable helix propensities compared to Hfl.

Scheme 1. Chemoenzymatic Synthesis of Hfl and Qfl.



An ideal fluoro-amino acid substitute for Leu should have the following characteristics. One, the overall shape of the amino acid needs to be similar to Leu, because Koksch has shown that the shape is important for substituting Leu residues in coiled coils.[16,17] Two, the helix propensity needs to be similar to Leu, or at least needs to be more favorable than Hfl. Unfortunately, the design of fluoro-amino acids with reasonable helix propensity remains difficult[15] despite the extensive knowledge of the main determinants for helix propensity.[18−23] Three, the amino acid needs to be readily synthesized in enantiomerically pure form. Four, the amino acid needs to be readily incorporated into peptides through solid-phase peptide synthesis. Toward fulfilling these needs, herein we report the gram scale stereoselective synthesis of both Hfl and a novel fluorinated amino acid (*S*)-5,5,5′,5′-tetrafluoroleucine (Qfl, Figure 1), the incorporation of Qfl into peptides, and the helix propensity of Qfl.

The difluoromethyl group (CF_2H) has been exploited in the development of bioactive compounds.[24−31] This group has been used as a bioisostere for the hydroxyl group because of similar hydrogen bond donating capability.[32] Nonetheless, the CF_2H group was incorporated as difluoromethionine into the interior of a protein to probe the environment near the fluorines,[33] demonstrating the compatibility of CF_2H with the hydrophobic core of proteins. If two CF_2H groups were introduced to give Qfl, the overall shape of Qfl would be similar to Leu without introducing any additional stereocenter. Furthermore, the size of Qfl would be in between that of Leu and Hfl, both of which are compatible with helical proteins.[3,6,8,9]

We synthesized Hfl and Qfl on gram scale using a chemoenzymatic approach[15] (Scheme 1). The corresponding acetones were reacted with the ylide in a Wittig reaction to give the unsaturated pyruvate esters (**1a** and **1b**). For synthesizing **1a**, we consistently obtained the side product **5** (≥30%) (Figure 2), despite performing the reaction according to literature procedures (1 equiv of hexfluoroacetone, 4.0 M ylide in tetrahydrofuran, 55 °C, vacuum distillation).[12] Hexafluoroacetone gas and hexafluoroacetone hexahydrate gave virtually the same results. To minimize this side product, we changed the equivalents of hexafluoroacetone, concentration of the ylide, solvent, and reaction temperature. By using 1.5 equiv of hexafluoroacetone under dilute conditions (0.4 M ylide) in benzene at 40 °C, the desired product was obtained with >99% purity upon vacuum distillation with 65% isolated yield. Either more equivalents of hexafluoroacetone or lower reaction temperatures resulted in a slight drop in isolated yield, whereas

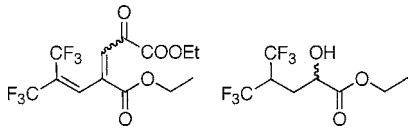


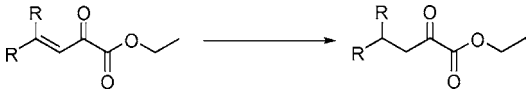
Figure 2. Chemical structures of two side products in the chemoenzymatic synthesis of Hfl.

(15) Chiu, H.-P.; Suzuki, Y.; Gullickson, D.; Ahmad, R.; Kokona, B.; Fairman, R.; Cheng, R. P. *J. Am. Chem. Soc.* **2006**, *128*, 15556−15557.
(16) Jäckel, C.; Salwiczek, M.; Koksch, B. *Angew. Chem., Int. Ed* **2006**, *45*, 4198−4203.
(17) Jäckel, C.; Seufert, W.; Thust, S.; Koksch, B. *ChemBioChem* **2004**, *5*, 717−720.
(18) Creamer, T. P.; Rose, G. D. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 5937−5941.
(19) Yang, A.-S.; Honig, B. *J. Mol. Biol.* **1995**, *252*, 351−365.
(20) Luo, P.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4930−4935.
(21) García, A. E.; Sanbonmatsu, K. Y. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 2782−2787.
(22) Vila, J. A.; Ripoll, D. R.; Scheraga, H. A. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13075−13079.
(23) Avbelj, F.; Luo, P.; Baldwin, R. L. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 10786−10791.
(24) Wang, E. A.; Walsh, C. *Biochemistry* **1981**, *20*, 7539−7546.
(25) Bey, P.; Gerhart, F.; van Dorsselaer, V.; Danzin, C. *J. Med. Chem.* **1983**, *26*, 1551−1556.
(26) Imperiali, B.; Abeles, R. H. *Biochemistry* **1986**, *25*, 3760−3767.
(27) Yamazaki, T.; Haga, J.; Kitazume, T. *Bioorg. Med. Chem. Lett.* **1991**, *1*, 271−276.
(28) Pu, Y. M.; Torok, D. S.; Ziffer, H.; Pan, X.-Q.; Meshnick, S. R. *J. Med. Chem.* **1995**, *38*, 4120−4124.
(29) Narjes, F.; Koehler, K. F.; Koch, U.; Gerlach, B.; Colarusso, S.; Steinkühler, C.; Brunetti, M.; Altamura, S.; De Francesco, R.; Matassa, V. G. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 701−704.
(30) Xu, Y.; Qian, L.; Pontsler, A. V.; McIntyre, T. M.; Prestwich, G. D. *Tetrahedron* **2004**, *60*, 43−49.
(31) Peleg, S.; Petersen, K. S.; Suh, B. C.; Dolan, P.; Agoston, E. S.; Kensler, T. W.; Posner, G. H. *J. Med. Chem.* **2006**, *49*, 7513−7517.
(32) Erickson, J. A.; McLoughlin, J. I. *J. Org. Chem.* **1995**, *60*, 1626−1631.
(33) Vaughan, M. D.; Cleve, P.; Robinson, V.; Duewel, H. S.; Honek, J. F. *J. Am. Chem. Soc.* **1999**, *121*, 8475−8478.
(34) Blaszczak, L. C.; McMurry, J. E. *J. Org. Chem.* **1974**, *39*, 258−259.
(35) Asano, Y.; Yamada, A.; Kato, Y.; Yamaguchi, K.; Hibino, Y.; Hirai, K.; Kondo, K. *J. Org. Chem.* **1990**, *55*, 5567−5571.
(36) Krix, G.; Bommarius, A. S.; Drauz, K.; Kottenhahn, M.; Schwarm, M.; Kula, M.-R. *J. Biotechnol.* **1997**, *53*, 29−39.

higher ylide concentrations produced the side product along with the desired product. Accordingly, compound **1b** was synthesized using these optimized conditions.

Compound **1a** was then subjected to hydrogenation conditions to reduce the alkene bond as described in the literature.[12] However, we obtained either unreacted reactant **1a** or the over hydrogenated alcohol (**6**, Figure 2) in the presence of the desired product **2a** (Table 1, entries 1 and 2). Changing

Table 1. Reduction of Fluorine-Containing Unsaturated Pyruvate Esters



entry	R	conditions	time (h)	product ratio[a] reactant:desired:side
1	CF_3	10 psi H_2, Pd/C	0.5	15:79:6
2	CF_3	10 psi H_2, Pd/C	1.5	0:79:21
3	CF_3	$TiCl_3$(aq)	0.25	2:98:0
4	CF_2H	$TiCl_3$(aq)	0.5	22:39:39
5	CF_2H	$TiCl_3$(aq)	8.0	26:53:21
6	CF_2H	10 psi H_2, Pd/C	0.75	0:93:7

[a] Determined by GC-MS.

the reaction time or H_2 pressure did not improve the exclusive production of the desired product **2a**. Interestingly, McMurry and co-workers have reported the selective reduction of alkene groups in enedicarbonyl compounds using $TiCl_3$(aq).[34] This reaction seemed particularly suitable for selectively reducing electron-deficient alkenes, such as those in compounds **1a** and **1b** due to the presence of multiple electron-withdrawing fluorines and the conjugated carbonyl. Compound **1a** was selectively reduced to **2a** by using $TiCl_3$(aq) nearly quantitatively (Table 1, entry 3). However, reducing the number of fluorines from 6 (R = CF_3) to 4 (R = CF_2H) resulted in significantly lower yields (Table 1, entries 4 and 5), showing that $TiCl_3$(aq) is only suitable for selectively reducing highly electron-deficient alkenes. As such, we performed hydrogenation on compound **1b** to give the desired product **2b** (Table 1, entry 6) instead.

Upon hydrolyzing compound **2a**, reductive amination to give Hfl (**3a**) was performed with NADH mediated by phenylalanine dehydrogenase,[15,35,36] which is known to operate on a variety of hydrophobic hydrocarbon side chains differing in size. The reductive amination proceeds through in situ formation of an imine between the α-keto acid and the ammonium in the buffer, followed by enantioselective imine reduction with NADH mediated by phenylalanine dehydrogenase. Since quantitative consumption of NADH would not be economical, formate hydrogenase was added to regenerate NADH with concomitant sacrificial oxidation of the formate in the buffer.[15,35,36] Surprisingly, phenylalanine dehydrogenase also converted the polar tetrafluoroleucine pyruvate into Qfl (**3b**) with 98% enantiomeric excess (Figure 3A), showing unprecedented tolerance to the polar CF_2H functionality. The high polarity of Qfl is

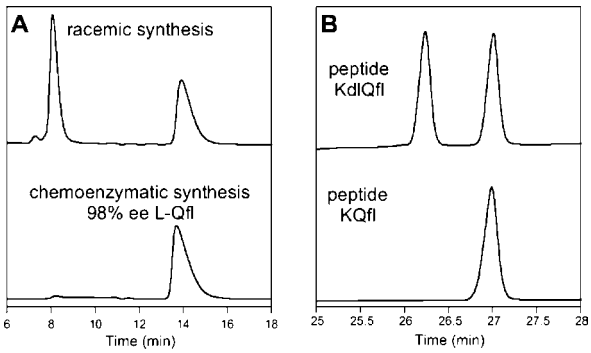


Figure 3. HPLC chromatograms for dl-Qfl obtained from racemic synthesis and Qfl from enzymatic reductive amination as eluted off a CrownPak CR(+) column (Panel A) and for peptides KdlQfl and KQfl synthesized from dl-Qfl and Qfl, recpectively, as eluted off a C_{18} reverse phase column (Panel B).

reflected in the short retention time (t_R) as eluted off a C_{18} reverse phase high performance liquid chromatography (HPLC) column (Table 2). Based on the t_R, the hydrophobicity of the Leu analogues followed the trend Hfl > Leu > Qfl. The *S* configuration for the Cα center was confirmed based on enantiomeric preference of phenylalanine dehydrogenase,[35,36] convention of the elution profile for the CrownPak CR(+) column,[37] and the Clough−Lutz−Jirgenson rule on how specific rotation changes with pH.[38] The backbone nitrogen was protected with Fmoc (9-fluorenylmethyloxycarbonyl) to afford the appropriately protected amino acid for solid-phase peptide synthesis. Importantly, all transformations including the enzymatic reductive amination were used to synthesize the amino acids on gram scale.

Table 2. Reverse Phase HPLC Retention Time (t_R), Helix Propensity (w), and Helix Forming Energetics (ΔG) for Qfl, Hfl, and Leu

amino acid	t_R (min)[a]	w	ΔG (kcal·mol^{-1})[b]
Qfl	4.8	0.445 ± 0.052	0.440 ± 0.068
Hfl	13.3	0.128 ± 0.023	1.12 ± 0.11
Leu	8.4	1.06 ± 0.12	-0.0317 ± 0.0654

[a] Retention time as eluted off an analytical C_{18} reverse phase column. [b] $\Delta G = -RT\cdot\ln(w)$.

The sequence of peptide KQfl, Ac-YGGKAAAA-KA**X**-AAKAAAAK-NH_2 (X = Qfl), was designed based on peptides studied by Baldwin for measuring helix propensity.[15,39] The peptide KQfl was synthesized by standard protocols for solid-phase peptide synthesis (SPPS), using Fmoc-based chemistry.[40] The identity of the peptide was confirmed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF), despite concerns

regarding repeated use of piperidine for Fmoc removal during peptide synthesis in the presence of the acidic protons on the CF_2H groups. Also, we independently synthesized and incorporated the racemic dl-Qfl to give peptide KdlQfl to check for racemization of the Cα center during peptide synthesis. Based on C_{18} reverse phase HPLC chromatograms (Figure 3B), peptide KdlQfl exhibited two peaks, both of which gave the same *m*/*z* by MALDI-TOF as peptide KQfl. Furthermore, the retention time of the trailing peak matched that for peptide KQfl. Importantly, Qfl was incorporated into peptides by SPPS without any protecting group, and the stereochemistry of the Cα center remained intact in the process.

Peptide KQfl was monomeric in solution based on sedimentation equilibrium experiments by analytical ultracentrifugation.[41] Therefore, circular dichroism (CD) spectra of KQfl should accurately reflect the helical content in the absence of any intermolecular interactions. The CD spectra of KQfl showed helical content between peptides KLeu and KHfl (Figure 4). The helix propensity (*w*) was calculated

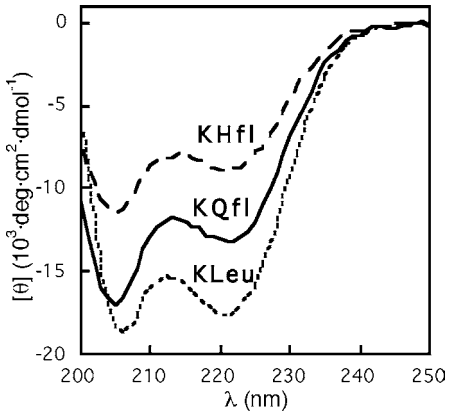


Figure 4. Circular dichroism spectra of peptides KQfl, KHfl, and KLeu at pH 7 (273 K) in 1 mM phosphate, borate, and citrate with 1 M NaCl.

from the CD data by using modified Lifson−Roig theory[15,42,43] (Table 2). Although the helix propensity of Qfl is half of that for Leu, the helix-forming energetics for Qfl is more favorable than that for Hfl by 0.68 kcal·mol^{-1} (Table 2). The favorable helix propensity for Leu can be attributed to the shielding of the helix hydrogen bond from water by the hydrophobic side chain,[20] resulting in a more stable helix hydrogen bond and thus more stable helix conformation. The low helix propensity of Hfl may be due to partial or full burial of the hydrophobic fluorocarbon side chain in the unfolded state with more exposure of this hydrophobic side chain in the helix state,[15] leading to unfavorable helix formation energetics. Apparently, the high polarity of the Qfl side chain would avoid the energetic penalty for side chain burial in the unfolded state as speculated for Hfl,[15] but would not provide shielding of the helix hydrogen bond from water like Leu.[20] Nevertheless, the helix propensity of Qfl is the highest among all fluoro-amino acids measured to date.[15]

We have developed a short and facile stereoselective chemoenzymatic synthesis for both Hfl and Qfl on gram scale. $TiCl_3$(aq) was used to selectively reduce a highly electron-deficient alkene for the synthesis of Hfl. Phenylalanine dehydrogenase is surprisingly capable of producing the polar Qfl despite the inherent preference for hydrophobic substrates.[35,36] This result may lead to further utility of this enzyme in synthesizing amino acids with polar side chains as well as hydrophobic ones. Furthermore, Qfl can be readily incorporated into peptides by standard protocols for solid-phase peptide synthesis without any side chain protection. Importantly, the helix formation energetics of Qfl is not only more favorable compared to Hfl by 0.68 kcal·mol^{-1}, but is the most favorable for all fluoro-amino acids measured to date.[15] The availability, ease of incorporation, and favorable helix propensity of Qfl sets the stage for future studies to probe if the dipole moment and hydrogen bonds involving the fluorines are important factors in the fluoro-stabilization effect of more than 1.5 kcal·mol^{-1}·residue^{-1}.[7,15] Furthermore, Qfl expands the repertoire of α-amino acids for peptide/protein design, and provides a facile means to introduce CF_2H groups for developing bioactive compounds.

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Supporting Information Available: Experimental details for the synthesis and characterization of tetrafluoroleucine and peptide KQfl, and experimental methods for circular dichroism spectroscopy and calculating the helix propensity of Qfl. This material is available free of charge via the Internet at http://pubs.acs.org.

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(37) Hilton, M.; Armstrong, D. W. *J. Liq. Chromatogr.* **1991**, *14*, 9−28.
(38) Greenstein, J. P.; Winitz, W. D. In *Chemistry of the Amino Acids*; John Wiley and Sons: New York, 1961; pp 1879−2155.
(39) Chakrabartty, A.; Kortemme, T.; Baldwin, R. L. *Protein Sci.* **1994**, *3*, 843−852.
(40) Fields, G. B.; Noble, R. L. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214.
(41) See the Supporting Information.
(42) Doig, A. J.; Chakrabartty, A.; Klingler, T. M.; Baldwin, R. L. *Biochemistry* **1994**, *33*, 3396−3403.
(43) Andersen, N. H.; Tong, H. *Protein Sci.* **1997**, *6*, 1920−1936.