5-Fluorinated L-Lysine Analogues as Selective Induced Nitric Oxide Synthase Inhibitors

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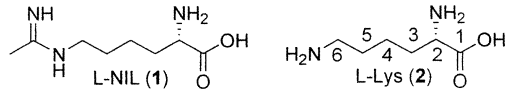
5(*S*)-Fluoro-*N*6-(iminoethyl)-L-lysine **(14)**, an analogue of the potent, selective induced nitric oxide synthase (iNOS) inhibitor iminoethyl-L-lysine (**1**), was synthesized and found to be a selective iNOS inhibitor.

Introduction

Elevated levels of nitric oxide (NO) generated by the action of induced nitric oxide synthase (iNOS) on L-arginine causes cellular cytotoxicity and tissue damage and are thought to contribute to the pathophysiology of a number of human diseases.[1] During the course of our research on inhibitors of iNOS, (iminoethyl)-L-lysine (NIL, **1**),[2] an analogue of L-lysine (**2**), was identified as



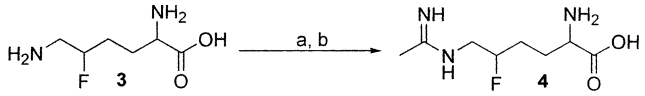
a potent, selective inhibitor of the iNOS isoform.[3] Selective iNOS inhibitors, such as L-NIL,[4] have been shown to suppress the increase in plasma nitrites and/or paw swelling associated with the overproduction of NO in animal models of acute and chronic inflammation including nonlethal endotoxemia,[5,6] carrageenan-induced paw edema,[6] and adjuvant-induced arthritis.[7,8]

We sought to optimize the activity observed with L-NIL. It is well-known that the introduction of fluorine into a bioactive molecule can significantly modulate its activity.[9] In our investigation of NIL analogues, introduction of fluorine at C-4 and C-5 as well as fluoroalkyl at C-6 was of particular interest. Installing fluorine at C-4 should have the least impact on the polar functionalities at C-2 and C-6.[9] Whereas, the introduction of fluorine at C-5 should reduce the basicity of the C-6 amidine. However, NOS inhibitors, albeit neuronal selective, have been reported with reduced basicity at the δ-guanidine of arginine such as δ-nitroarginine.[10] The effect of the introduction of fluorine into the lysine side chain of NIL at C-5 on iNOS inhibition is described here.

Synthesis

Commercially available 5-fluoro-D,L-lysine **3**[11] allowed a quick look at iNOS inhibition. As shown in Scheme 1, 6-(iminoethyl)-5-fluoro-D,L-lysine **4** was synthesized using methodology described previously.[2] In Table 1, **4** is shown to have iNOS inhibition activity comparable to that of L-NIL. For this reason, separation of the four isomers of 5-fluorolysine was undertaken in order to identify the active isomer.

Scheme 1[a]



[a] (a) $CuCO_3$, H_2O, Δ, 1 h. (b) pH 9−9.5, ethyl acetimidate hydrochloride, ion exchange, reverse phase chromatography.

Table 1. Bioassay Data[3]

	NOS inhibition (IC_{50}, μM) or % inhibition at 10 μM			selectivity	
compd	iNOS	eNOS	nNOS	iNOS vs eNOS	iNOS vs nNOS
NIL	4.9 ± 1.65 μM	138 μM	35 μM	28	7
4	7.84 μM	182 μM	70.4 μM	23	9
14 (2*S*,5*S*)	2.36 μM	81 μM	21.7 μM	34	9
15 (2*S*,5*R*)	4.0%	28.2%	0.00%		
16 (2*R*,5*S*)	47.8 μM	374 μM	311 μM	8	7
17 (2*R*,5*R*)	8.9%	6.52%	18%		
27	21.4 μM	139 μM	140 μM	7	7
32	8.75 μM	57.5 μM	33.8 μM	7	4

Initially, as shown in Scheme 2, the *N*,*N*-α,ϵ-bis-(benzyloxycarbonyl)-5-fluorolysine **5**, which was synthesized under standard conditions, was subjected to chiral chromatography using Chiralpak OJ. The initial separation yielded three of the four isomers. The repeat separation of two coeluting isomers gave the fourth isomer. This process resulted in isomers **6**−**9**. The shortcoming of this method of chiral chromatography was that the use of alcohol in the presence of acid as an eluting solvent resulted in partial esterification. The protecting groups were removed under catalytic hydrogenation conditions, which resulted in α-amino acids **10**−**13**. Each isomer was treated as described for **4** to give amidines **14**−**17**. As shown in Table 1, the *S*,*S*-isomer **14** had much of the iNOS enzyme inhibition activity. Because **14** had a desirable profile of enzyme inhibition, we explored alternative routes to **14** using different protecting group strategies. In Scheme 3, the separation of the *N*,*N*-α,ϵ-diphthaloyl-5-fluorolysine methyl ester **18**[12] is illustrated. Using Chiralpak AD chromatography conditions as shown in Scheme 3, the isomers **19**−**22** were separated to give three of the four isomers on the initial separation. Hydrolysis to the

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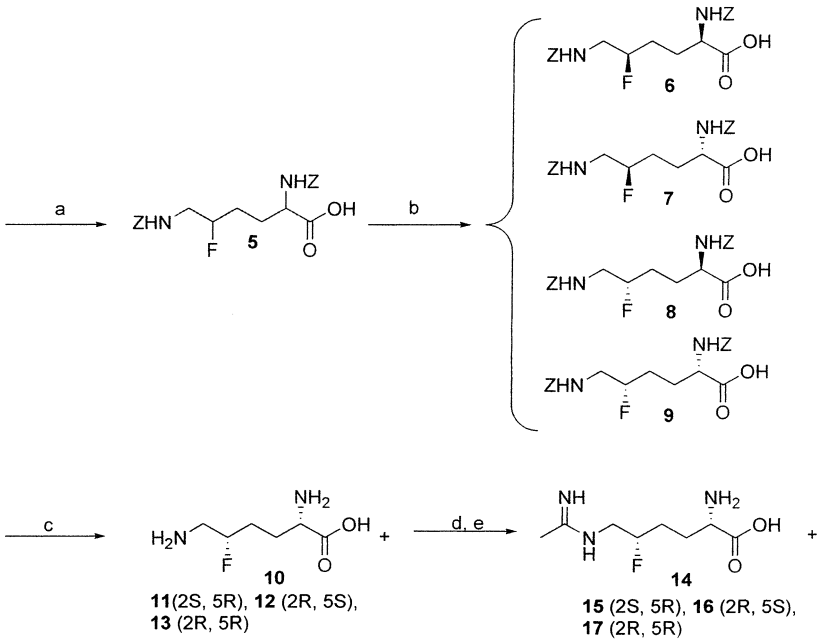
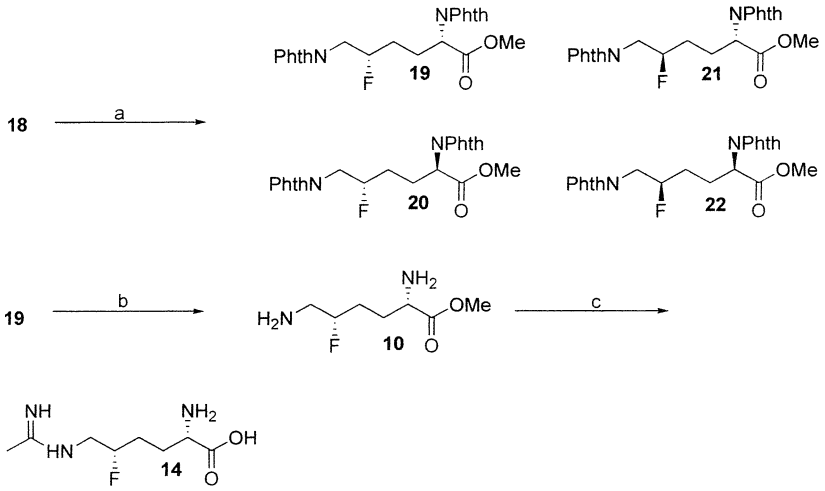
Scheme 2[a]



[a] (a) Compound **3**, dibenzyl dicarbonate, $NaHCO_3$, acetone/H_2O (3:2), rt, overnight, 87%. (b) Chiralpak OJ, heptane, EtOH, MeOH, TFA. (c) H_2, 5 psi, 5% Pd/C, HOAc, 95%. (d) $CuCO_3$, H_2O, Δ, 1 h. (e) pH 9−9.5, ethyl acetimidate hydrochloride, ion exchange chromatography, reverse phase chromatography.

Scheme 3[a]

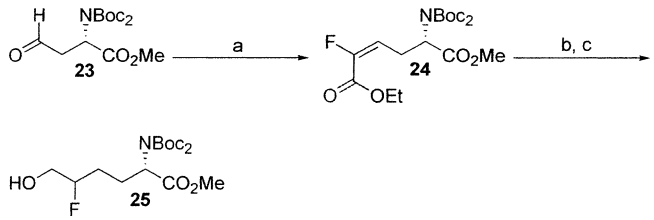


[a] (a) Chiralpak AD, MeOH. (b) HCl/HOAc, Δ. (c) See Scheme 1 for reaction conditions.

amino acids gave **10**−**13**. By capillary electrophoresis, the product of hydrolysis of **19** gave the desired amino acid isomer **10**, which was taken onto **14**. The desired isomer **19** was readily separated from isomers **20**−**22**. To improve the yield of **19**, isomer **20** was treated with DBU in CH_2Cl_2 to epimerize the α-amino acid stereocenter to provide additional amounts of **19**. The mixture was rechromatographed to provide additional quantities of **19**.

Another synthetic approach explored the use of aspartyl semialdehyde.[13] The synthetic strategy followed that described by Taguchi et al.,[14] in which they synthesized 5-fluoro-L-lysine with the α-amino acid stereocenter set by the chiral starting material. However, their synthesis commenced with mono-t-Boc-L-homoserine methyl ester. Our concern about this starting material was retaining the stereochemical integrity at the α-amino acid stereocenter; also, the possibility

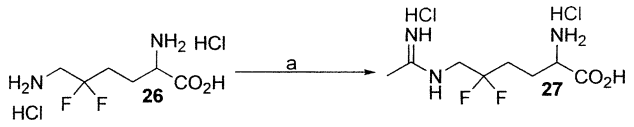
Scheme 4[a]



[a] (a) Triethyl 2-fluoro-2-phosphonoacetate, NaH, −40 °C to rt, 60%. (b) H_2, Pd/C, EtOH. (c) $NaBH_4$, MeOH.

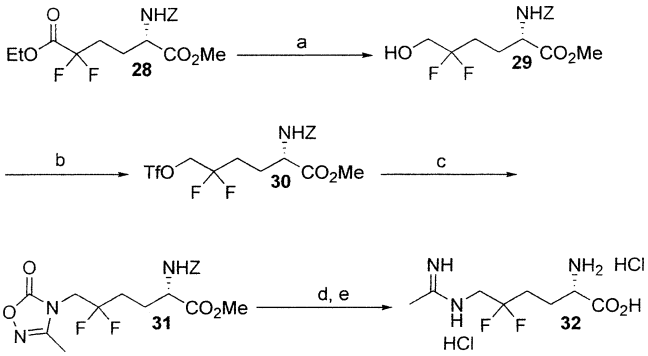
of lactone formation existed. Therefore, the bis-Boc-aspartyl semialdehyde **23**[13] was used to explore the viability of the Taguchi approach. The first three steps of the synthesis are illustrated in Scheme 4. The use of Horner−Emmons chemistry homologated the amino acid side chain and introduced the desired 5-fluoro functionality and the correct chain length to give **24**.

Scheme 5[a]



[a] (a) ethyl acetimidate hydrochloride, $CuCO_3$, ion exchange resin.

Scheme 6[a]



[a] (a) $NaBH_4$, MeOH; (b) triflic anhydride, DCM, DMAP. (c) 3-methyl-[1,2,4]oxadiazol-5-one potassium salt, DMF. (d) 5% Pd/C, MeOH; (e) 2 N HCl.

The alkene was reduced under catalytic hydrogenation conditions and the ester subsequently reduced with sodium borohydride, giving **25**. This illustrates that the bis-protected amine approach works in very much the same fashion as the monoprotected amine described previously and is a reasonable approach to the synthesis of 5-fluoro-L-lysine. The appeal of this route was that only a diastereomeric pair required separation.

In parallel with synthesis of 5-fluoro amidine **14**, the synthesis of 5,5-difluoro amidine **27** was investigated. The achiral synthesis of 5,5-difluorolysine **26** has been reported previously.[15] As depicted in Scheme 5, the amidine **27** was synthesized in the manner described in Scheme 1. Although **27** has modest inhibitory activity, we explored a chiral synthesis of 5,5-difluoro-L-lysine as represented in Scheme 6. Our approach made use of the electron-transfer chemistry of Taguchi et al.[16] Protected L-vinylglycine sets the amino acid stereochemistry and is commerically available.[17] Ethyl difluoroiodoacetate,[18] which is used to homologate the vinylglycine, adds the necessary length and the *gem*-difluoro moiety, yielding **28**. This reaction is somewhat capricious and the yields are variable. Burton chemistry[19] was attempted with results similar to that described for Taguchi chemistry. The ester is reduced with sodium borohydride, yielding alcohol **29**. The triflate **30** is synthesized by employing triflic anhydride in the presence of DMAP. Subsequently, the triflate is displaced by the potassium salt of 3-methyl-[1,2,4]oxadiazol-5-one[20] to give the protected amidine **31**. Two methods to expose the amidine were investigated, either zinc in acetic acid or catalytic hydrogenation.[20] Catalytic hydrogenation was used to unmask the amidine and to remove the benzyloxycarbonyl protecting group. The methyl ester was removed under acid hydrolysis conditions to give the chiral 5,5-difluoro-L-NIL **32**.

Bioassay Results

Enzyme inhibition assays were performed with the human NOS isoforms as described previously.[4] 5(*S*)-Fluoro-L-**NIL 14** was a more potent inhibitor of iNOS isozyme than L-NIL, as shown in Table 1. In addition to **14**, only 5(*R*)-fluoro-L-NIL **16** showed very modest activity as an iNOS inhibitor The 5,5-difluoro-L-NIL amidine **32** did not have the potency nor the selectivity when compared to L-NIL. 5(*S*)-Monofluoro-L-NIL **14** is more potent than L-NIL and demonstrates comparable selectivity.

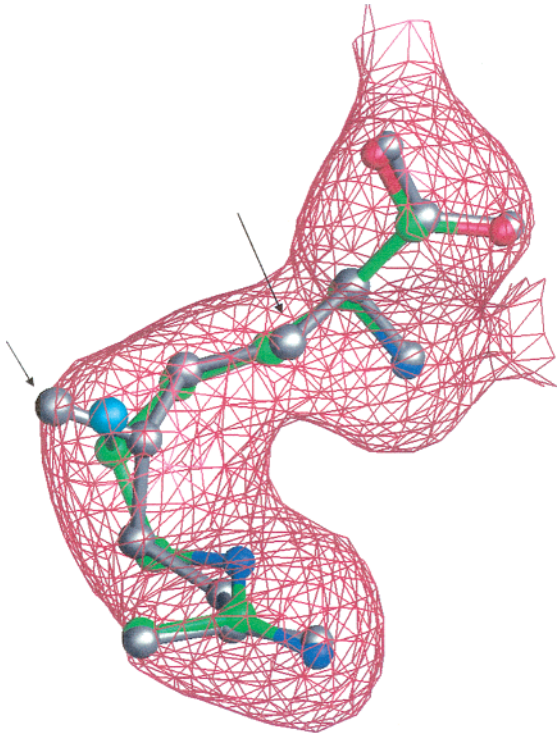


Figure 1. The refined *S*-configuration (in normal conventional atom color, O = red, N = blue, C = green, and F = cyan) and *R*-configuration (in silver) structures of compound **14** dock into the unbiased, i.e., without inhibitor contribution, difference electron density map. As indicated by the arrows, the F- and C_b-atoms of the *R*-configuration are partially out of the contour map, while those of the *S*-configuration are still in.

X-ray Crystallography

Amidine **14**, a single isomer, has been cocrystallized with the oxygenase domain of mouse iNOS (residues 66−498). The protein purification and crystal preparation were described by Tainer et al.[21] The X-ray diffraction data were collected at the IMCA beamline 17ID of the Advanced Photon Source of the Argonnne National Laboratory. The crystals diffract to 2.34 Å resolution. Since the absolute configuration around the fluorine atom was not known, the inhibitor coordinates with both configurations (*S*- and *R*-) are refined separately with the protein. The final refinement statistics did not suggest which isomer was in the crystal. However, the unbiased difference electron density (Figure 1, without the contribution of inhibitor molecule) indicated that the *S*-enantiomer had a slightly better fit than the *R*-configuration. On the basis of these data, amidine **14** was assigned the 2*S*,5*S*-stereochemistry.

There are several dominant features in the inhibitor-binding pocket, as shown in Figure 2. Two strands wrap around the inhibitor. The lower strand, FNGWYM (the side chains of the starting residue Phe363 and the ending residue Met368 are shown in the figure), covers

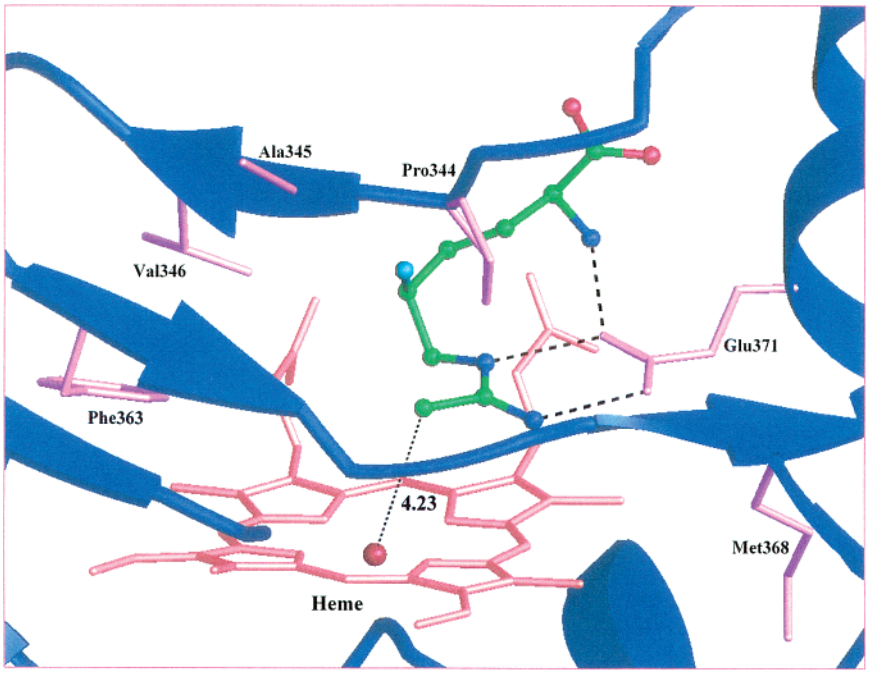


Figure 2. This is a ribbon representation of the inhibitor-binding pocket. The protein is shown in deep blue ribbons. The side chains of some of the neighboring residues are shown in pink. Only the beginning residue, Phe363, and the ending residue, Met368, of the lower strand are shown for the sake of clarity. Heme is in orange. The inhihitor, compound **14**, is depicted in the conventional color: O = red, N = blue, C = green, and F = cyan.

the amidine group. The upper strand, Pro344-Ala345-Val346, houses a suitable hydrophobic pocket in which to fit the fluorine atom. Another dominant feature is the formation of three hydrogen bonds between the inhibitor and Glu371 of the protein. This type of hydrogen-bond formation is universal for all guanidine or amidine inhibitors of NOS enzymes in all of the structures published to date. In this case, the amidine group of the inhibitor binds to the carboxylate group of Glu371 with two hydrogen bonds. The amino acid end of the inhibitor curves back to orient the amino group in a proper position to form a rather strong hydrogen bond with Glu371. The heme iron does not interact with the inhibitor. The distance between iron and the closest methyl group of the inhibitor is 4.23 Å. It is not shown in the figure, but the amino acid end of the inhibitor is surrounded by a cluster of polar residues, Arg260, Arg382, Asn257, Asp376, etc. Although the heteroatoms (polar atoms) of the inhibitors, **14** and NIL shown in Figure 3, superimpose well in the structure, their overall conformations are quite different. The conformational differences of **14** and NIL are likely due to the substitution of fluorine at the middle of the chain and due to the affinity of fluorine to the hydrophobic residues Pro344-Ala345-Val346.

Conclusion

In this paper, we have described the synthesis of 5(*S*)-fluoro-L-**NIL 14** and 5,5-difluoro-L-NIL **32.** The introduction of two fluorines on C-5 of L-NIL yields **32**, which is not as selective as L-NIL. This would seem to suggest that amelioration of the bascity of the amidine of **32** by the introduction of two fluorines at C-5 results in the loss of NOS enzyme selectivity. However, the introduction of a single fluorine into the side chain of L-NIL gave amidine **14**, which is a potent, selective iNOS inhibitor.

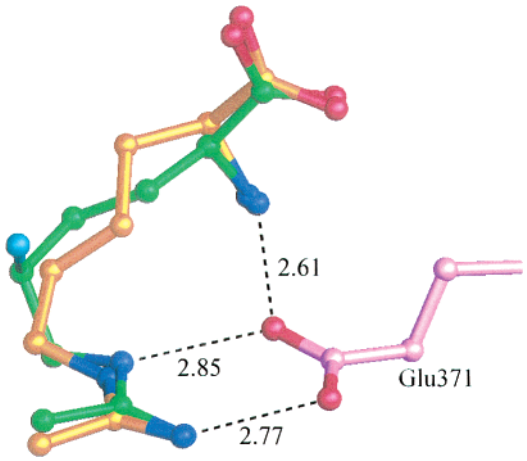


Figure 3. This is the blow-up picture of Figure 2. NIL molecule is added. In the NIL molecule, all its carbon atoms are depicted as yellow.

Additional biological assessment of **14** will be described elsewhere.

Experimental Section

Thin-layer chromatograms were run on 0.25 mm EM precoated plates of silica gel 60 F254. Visualization was achieved by exposure to I_2 or phosphomolybdic acid or $Ca(ClO)_2$ followed by spraying with a 0.5% KI 0.5% potato starch solution. GC was run on a Shimadzu 9A using capillary columns from several vendors. Preparative column chromatograpy using flash chromatography conditions was performed on EM silica gel or on a Biotage Flash-40 system. To successfully use the Biotage columns under flash chromatography conditions, the optimal R_f needs to be 0.15−0.2, in contrast to an R_f of 0.35−0.4 as described by Still.[22] Reverse-phase chromatography was performed on a Rainin preparative HPLC system with a YMC ODS-AQ (10 μm) preparative column eluting with acetonitrile/water (0.05% HOAc) at 10 mL/min. HPLC chromatography was performed using a Hewlitt-

Packard HP 1100 with a dual absorbance detector. Both chiral and achiral columns were acquired from several vendors. Capillary electropheresis analyses were performed on a Beckman Coulter system. ^{1}H NMR spectra were taken in commercially available deuterated solvents on a Bruker-400 MHz Ultrashield. All chemical shifts are reported in parts per million (δ) downfield (positive) relative to Me_4Si (organic solvents) or 3-(trimethylsilyl)propionic-*2,2,3,3-d*$_4$ acid, sodium salt (D_2O) as an internal standard for ^{1}H and ^{13}C NMR. ^{13}C NMR spectra were obtained on a Bruker-400 MHz Ultrashield spectrometer at an observation frequency of 100 MHz. The observation frequency for ^{19}F NMR was 376 MHz, and the chemical shifts in parts per million (δ) are reported upfield or downfield relative to fluorotrichloromethane (Freon-11). ^{19}F NMR spectra were proton decoupled. All reagents were purchased from Sigma-Aldrich, except where noted, and used as is. Solvents were purchased from Burdick & Jackson. Microanalyses and optical rotations were performed in-house. The optical rotations were performed at concentrations of 2 mg/mL in $CHCl_3$ for the protected amino acids and MeOH for the amino acids.

***N*^{6}-Iminoethyl-5-fluorolysine Hydrochloride (4).** Starting with 5-fluorolysine **3**,[11] amidine **4** was prepared in the manner described in the literature:[1] ^{1}H NMR (400 MHz, CD_3OD) δ 1.77−2.22 (m, 4 H), 2.27 (s, 3 H), 3.48−3.78 (m, 2 H), 4.02−4.12 (m, 1 H), 4.69−4.89 (m, 1 H); ^{13}C NMR (100 MHz, CD_3OD) δ 19.4, 27.6, 29.5 (d, J = 20.3 Hz), 47.7 (d, J = 21.0 Hz), 53.6, 92.1 (d, J = 173 Hz), 167.5, 171.8; ^{19}F NMR −187.5.

5-Fluoro-*N*^{2},*N*^{6}-bis(benzoxycarbonyl)lysine (5). To a stirred solution of 5-fluorolysine (3.70 g, 18.4 mmol) in 100 mL of acetone:H_2O (2:3) was added dropwise a solution of dibenzyl dicarbonate (13.20 g, 46.1 mmol) in 40 mL of acetone. After stirring 18 h, the reaction was concentrated to remove the acetone. The aqueous layer was washed with EtOAc (1 × 100 mL), neutralized to pH 3, and extracted with EtOAc (2 × 100 mL). After neutralization, the combined organic layers were washed with saturated brine, dried over anhydrous Na_2SO_4, and concentrated to yield 8.3 g of crude product **5**. After purification on a Biotage system, 7.16 g (87%) of desired purified **5** was recovered: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.52−2.18 (m, 4 H), 3.20−3.55 (m, 2 H), 4.28−4.67 (m, 2 H), 5.02−5.28 (m, 4 H), 7.30−7.39 (m, 10 H). Anal. ($C_{22}H_{25}FN_2O_6$·0.50 H_2O) C, H, N.

(5*S*)-5-Fluoro-*N*^{2},*N*^{6}-bis(benzoxycarbonyl)-L-lysine (6). The product **5** (6.05 g) was subjected to chiral chromatography employing Chiralcel OJ and eluting with a quaternary mixture of heptane/MeOH/EtOH/TFA to yield 0.48 g (8%): $[\alpha]_D$ = +6.8 ± 3.5.

(5*R*)-5-Fluoro-*N*^{2},*N*^{6}-bis(benzoxycarbonyl)-L-lysine (7). See the method for compound **6**, which yielded 0.97 g (16%) of **7**: $[\alpha]_D$ = −7.5 ± 3.5; ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.51−2.18 (m, 4 H), 3.18−3.55 (m, 2 H), 4.28−4.65 (m, 2 H), 5.04−5.18 (m, 4 H), 7.28−7.40 (m, 10 H).

(5*S*)-5-Fluoro-*N*^{2},*N*^{6}-bis(benzoxycarbonyl)-D-lysine (8). See the method for compound **6**, which yielded 0.28 g (5%) of **8**.

(5*R*)-5-Fluoro-*N*^{2},*N*^{6}-bis(benzoxycarbonyl)-D-lysine (9). See the method for compound **6**, which yielded 0.53 g (9%) of **9**. $[\alpha]_D$= −13.3 ± 3.5. Anal. ($C_{22}H_{25}FN_2O_6$·1.1H_2O) C, H, N.

(5*S*)-5-Fluoro-L-lysine (10). The protecting groups were removed under catalytic hydrogenation conditions using 5% Pd/C in EtOH/HOAc to give a quantitative yield of the amino acid **10**: $[\alpha]_D$ = +9.3 ± 3.5; ^{1}H NMR (400 MHz, D_2O) δ 1.63−1.98 (m, 2 H), 2.02 (q, J = 8.0 Hz, 2 H), 3.04−3.30 (m, 2 H), 4.04 (t, J = 6.5 Hz, 1 H), 4.85 (tt, J = 3.4, 9.2 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O) δ 25.6 (d, J = 6 Hz), 27.7 (d, J = 20 Hz), 43.0 (d, J = 20 Hz), 52.7, 90.4 (d, J = 171 Hz), 172.0; ^{19}F NMR (376 MHz, D_2O, $CFCl_3$) δ −190.8.

(5*R*)-5-Fluoro-L-lysine (11). The experimental conditions used to generate **10** were used to produce the amino acid **11**: $[\alpha]_D$ = −23.4 ± 3.4; ^{1}H NMR (400 MHz, D_2O) δ 1.58−1.88 (m, 2 H), 1.89−2.01 (m, 1 H), 2.02−2.15 (m, 1 H), 3.03−3.25 (m, 2 H), 4.01 (t, J = 6.4 Hz, 1 H), 4.65−4.89 (m, 1 H); ^{13}C NMR (100 MHz, D_2O) δ 25.6 (d, J = 6 Hz), 27.7 (d, J = 20 Hz), 43.0 (d, J = 20 Hz), 52.7, 90.4 (d, J = 171 Hz), 172.0; ^{19}F NMR (376 MHz, D_2O, $CFCl_3$) δ −190.8. Anal. ($C_6H_{13}N_2O_2F$·2HCl·0.2H_2O) C, H, N.

(5*S*)-5-Fluoro-D-lysine (12). The experimental conditions used to generate **10** were used to produce the amino acid **12**: $[\alpha]_D$ = +10.7 ± 3.4; see compound **11** for NMR.

(5*R*)-5-Fluoro-D-lysine (13). The experimental conditions used to generate **10** were used to produce the amino acid **13**: $[\alpha]_D$ = −23.4 ± 3.3; see compound **10** for NMR.

(5*S*)-*N*^{6}-(Iminoethyl)-5-fluoro-L-lysine Hydrochloride (14). Amidine **14** was prepared in the same manner as described for **4**: ^{1}H NMR (400 MHz, D_2O, TSP) δ 1.69−1.89 (m, 2 H), 1.96−2.07 (m, 2 H), 2.16 (s, 3 H), 3.37−3.59 (m, 2 H), 4.03 (t, J = 6.4 Hz, 1 H), 4.79−4.85 (m, 1 H); ^{13}C (100 MHz, D_2O, TSP) δ 18.9, 25.9, 27.7 (d, J = 20 Hz), 45.8 (d, J = 20 Hz), 53.6, 91.8 (d, J = 169 Hz), 166.2, 172.1; ^{19}F (376 MHz, D_2O, $CFCl_3$) δ −187.5. Anal. ($C_8H_{16}N_3O_2F$·2HCl·2H_2O) C, H, N.

(5*R*)-*N*^{6}-(Iminoethyl)-5-fluoro-L-lysine Hydrochloride (15). Amidine **15** was prepared in the same manner as described for compund **4**: ^{1}H NMR (400 MHz, D_2O, TSP) δ 1.60−1.90 (m, 2 H), 1.90−2.02 (m, 1 H), 2.04−2.12 (m, 1 H), 2.16 (s, 3 H), 3.38−3.58 (m, 2 H), 4.02 (t, J = 6.4 Hz, 1 H), 4.79−4.86 (m, 1 H); ^{13}C (100 MHz, D_2O, TSP) δ 18.9, 25.8, 27.5 (d, J = 20 Hz), 45.8 (d, J = 21 Hz), 53.7 91.7 (d, J = 169 Hz), 166.2, 172.3; ^{19}F (376 MHz, D_2O, $CFCl_3$) δ −187.0. Anal. ($C_8H_{16}N_3O_2F$·2HCl·1.3H_2O) C, H, N.

(5*S*)-*N*^{6}-(Iminoethyl)-5-fluoro-D-lysine Hydrochloride (16). Amidine **16** was prepared in the same manner as described for compound **4**: see compound **15** for NMR data.

(5*R*)-*N*^{6}-(Iminoethyl)-5-fluoro-D-lysine Hydrochloride (17). Amidine **17** was prepared in the same manner as described for compound **4**: see compound **14** for NMR data.

Methyl 2,6-Bis(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-fluorohexanoic Acid (18). Diphthaloyl-5-fluorolysine methyl ester **18** was prepared as described in the literature:[12] ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.58−1.94 (m, 2 H), 2.34−2.60 (m, 2 H), 3.75 (s, 5 H), 3.90−3.99 (m, 1 H), 4.87−4.91 (m, 1 H), 7.70−7.90 (m, 8 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 24.8 (d, J = 25 Hz), 25.0 (d, J = 25 Hz), 30.0, 30.1, 41.9 (d, J = 23 Hz), 42.1 (d, J = 22 Hz), 52.2, 53.2, 90.2 (d, J = 165 Hz), 90.8 (d, J = 175 Hz), 123.7, 123.9, 132.0, 132.2, 134.5, 134.7, 167.8, 167.9, 168.2, 169.6; ^{19}F NMR (376 MHz, $CDCl_3$) δ −187.8, −188.3. Anal. ($C_{23}H_{19}N_2O_6F$) C, H, N.

Methyl (2*S*,5*S*)-2,6-Bis(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-fluorohexanoate (19). Compound **18** (4.3 g) was separated into four isomers using chiral chromatography on Chiralpak AD in 100% MeOH. This resulted in isomers **19**−**22**. The yield of isomer **19** was 0.90 g: $[\alpha]_D$ = 4.3 ± 3.4.

Methyl (2*R*,5*S*)-2,6-Bis(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-fluorohexanoate (20). See the method for compound **19**. The yield of isomer **20** was 0.28 g: $[\alpha]_D$ = −33.1 ± 3.4.

Methyl (2*S*,5*R*)-2,6-Bis(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-fluorohexanoate (21). See the method for compound **19**. The yield of isomer **21** was 0.25 g: $[\alpha]_D$ = +23.7 ± 3.4. Anal. ($C_{23}H_{19}N_2O_6F$·2H_2O) C, H, N.

Methyl (2*R*,5*R*)-2,6-Bis(1,3-dioxo-1,3-dihydro-2*H*-isoindol-2-yl)-5-fluorohexanoic Acid (22). See the method for compound **19**. The yield of isomer **22** was 0.45 g: $[\alpha]_D$ = −12.3 ± 3.4. Anal. ($C_{23}H_{19}N_2O_6F$·0.3H_2O) C, H, N.

Methyl 2-[Bis(*tert*-butoxycarbonyl)amino]-4-oxobutanoate (23). Bis-Boc-aspartyl semialdehyde **23** was prepared as described in the literature.[15]

1-Ethyl 6-Methyl (2*Z*,5*S*)-5-[bis(tert-butoxycarbonyl)-amino]-2-fluorohex-2-enedioate (24). Intermediate **24** was prepared in the same manner described by Taguchi et al.[14] with a yield of 60%: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.34 (t, J = 7.0 Hz, 3 H), 1.48 (s, 9 H), 1.49 (s, 9 H), 3.26 (dt, J = 1.5, 8.3 Hz, 2 H), 3.74 (s, 3 H), 4.29 (dq, J = 1.5, 7.0 Hz, 2 H), 5.02 (t, J = 7.5 Hz, 1 H), 5.93 (td, J = 8.3, 20.7 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.5, 27.0, 28.3, 52.7, 57.9, 61.9, 83.7, 119.0 (d, J = 21.4 Hz), 149.6, 153.0, 161.9, 171.6. Anal. ($C_{19}H_{30}NO_8F$) C, H, N.

Methyl *N*,*N*-Bis(*tert*-butoxycarbonyl)-5-fluoro-6-hydroxy-L-norleucinate (25). Step a. The alkene diester product **24** was reduced under catalytic hydrogenation conditions using 5% Pd/C as a catalyst. The yield of **25a** was 86%.

Step b. To a rapidly stirred solution of $NaBH_4$ (0.044 g, 1.2 mmol) in 5 mL of MeOH was added dropwise the product **25a** (0.42 g, 1.2 mmol) in 20 mL of MeOH. After stirring for 3 h at room temperature, additional $NaBH_4$ (0.016 g) was added. After 4 h, the reaction was concentrated under vacuum. The residue was dissolved in 25 mL of EtOAc and 25 mL of H_2O, the layers were separated, the aqueous layer was back-extracted with EtOAc (2 × 25 mL), and the combined organic layers dried over $MgSO_4$, filtered, and evaporated. The residue was purified on a Flash-40 system to yield 0.12 g (27%) of **25**. In addition, an equal amount of aldehyde hemiacetal was isolated: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.43 (s, 18 H), 1.45−1.78 (m, 2 H), 1.82−2.03 (m, 1 H), 1.82−2.03 (m, 1 H), 2.12−2.30 (m, 1 H), 3.58−3.62 (m, 1 H), 3.65 (s, 5 H), 4.43−4.64 (m, 1 H), 4.81 (dt, J = 4.8, 10.8 Hz, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.8, 25.9, 28.0 (d, J = 9 Hz), 28.3 (d, J = 9 Hz), 28.4, 52.6, 57.9, 58.3, 64.9 (d, J = 15 Hz), 65.2 (d, J = 15 Hz), 63.8, 94.8 (d, J = 169 Hz), 95.1 (d, J = 169 Hz), 152.4, 172.5. Anal. ($C_{17}H_{30}NO_7F$) C, H, N.

5,5-Difluorolysine (26). 5,5-Difluorolysine was synthesized according to the literature procedure.[15]

***N*^{6}-(Iminoethyl)-5,5-difluorolysine Dihydrochloride (27).** Amidine **27** was prepared as described for compound **4**: ^{1}H NMR (400 MHz, D_2O) δ 1.99−2.16 (m, 4 H), 2.18 (s, 3 H), 3.73 (t, J = 15 Hz, 2 H), 4.03 (t, J = 5 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O, TSP) δ 19.0, 22.5, 29.9 (t, J = 24 Hz), 45.9 (t, J = 27 Hz), 52.6, 122.2 (t, J = 242 Hz), 167.5, 172.0; ^{19}F NMR (376 MHz, D_2O, $CFCl_3$) δ −105.7

7-Ethyl 1-Methyl 2-[(Benzyloxycarbonyl)amino]-5,5-difluoroheptanedioate (28). To a stirred solution of methyl benzyloxycarbonyl-L-vinylglycinate (0.50 g, 2.0 mmol) in 10 mL of ultrapure DMF was added ethyl difluoroiodoacetate (0.65 g, 2.6 mmol) and Cu (0.04 g, 0.6 mmol). After 24 h, EtOAc (50 mL) was added to the reaction. The organic layer was washed with brine solution (5 × 50 mL), dried over $MgSO_4$, filtered, and concentrated under vacuum. The residue was dissolved in 20 mL of HOAc/EtOH (1:3) and treated with Zn (0.26 g, 3.9 mmol). After heating at 80 °C for 4 h, the reaction mixture was cooled to room temperature and concentrated under vacuum. To the residue was added 25 mL of EtOAc, which was washed with saturated $NaHCO_3$ (25 mL), brine (25 mL), and H_2O (25 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated to yield 0.54 g of crude product. After purification on a Flash-40 system, 0.25 g (32%) of **28** was isolated: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.35 (t, J = 7.5 Hz, 3 H), 1.81−1.90 (m, 1 H), 2.05−2.28 (m, 3 H), 3.78 (s, 3 H), 4.33 (q, J = 7.5 Hz, 2 H), 5.12 (s, 2 H), 5.36 (br d, 1 H), 7.30−7.40 (m, 5 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.3, 25.2, 30.9 (t, J = 23 Hz), 53.1, 63.4, 67.5, 116.0 (t, J = 251 Hz), 128.5, 128.8, 129.0, 136.4, 156.3, 164.2, 172.4; ^{19}F NMR (376 MHz, D_2O, $CFCl_3$) δ −105.6. Anal. ($C_{17}H_{21}NO_6F_2$) C, H, N.

Methyl 2-[(Benzyloxycarbonyl)amino]-5,5-difluoro-7-hydroxyheptanoate (29). Ester **28** was reduced in the same manner as described for compound **25**, step b, to yield **29**: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.86−2.08 (m, 3 H), 2.15−2.24 (m, 1 H), 3.65−3.79 (m, 5 H), 4.35−4.44 (m, 1 H), 5.00−5.15 (m, 2 H), 5.50 (br d, 1 H), 7.27−7.40 (m, 5 H).

Methyl 2-[(Benzyloxycarbonyl)amino]-5,5-difluoro-7-{[(trifluoromethyl)sulfonyl]oxy}heptanoate (30). To a stirred ice bath cooled solution of **29** (0.42 g, 1.3 mmol) in 20 mL of DCM was added DMAP (0.31 g, 2.5 mmol) followed by triflic anhydride (0.72 g, 2.5 mmol). When the triflic anhydride was added, there was a 15 °C temperature rise. After stirring for 2 h, the reaction was extracted with brine (20 mL) and H_2O (20 mL), dried over $MgSO_4$, filtered, and concentrated. The crude product was purified under Flash-40 chromatography conditions. The yield of **30** was 0.55 g (89%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.82−2.22 (m, 4 H), 3.77 (s, 3 H), 4.38−4.46 (m, 2 H), 4.50 (t, J = 11.4 Hz, 1 H), 5.11 (s, 2H), 5.46 (brd, 1 H), 7.34−7.39 (m, 5 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 25.2 (t, J = 4 Hz), 29.8 (t, J = 24 Hz), 53.2, 53.4, 67.7, 73.0 (t, J = 36 Hz), 119.5 (t, J = 243 Hz), 128.6, 128.8, 129.0, 136.4, 156.2, 172.3; ^{19}F NMR (376 MHz, $CDCl_3$) δ −71.5 (3 F), −105.7 (2 F).

Methyl *N*-(Benzyloxycarbonyl)-5,5-difluoro-6-(3-methyl-5-oxo-1,2,4-oxadiazol-4(5*H*)-yl)-L-norleucinate (31). To a solution of **30** (0.55 g, 1.2 mmol) in 20 mL of DMF was added potassium 3-methyl-[1,2,4]oxadiazol-5-one (0.18 g, 1.3 mmol). After heating for 20 h at 50 °C, the reaction was concentrated under high vacuum. The residue was dissolved in 25 mL of EtOAc, washed with brine (2 × 25 mL), dried $MgSO_4$, and concentrated under vacuum. The crude residue was purified by using a Flash-40 chromatography system. The yield of **31** was 0.20 g (42%): ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.88−2.08 (m, 3 H), 2.10−2.24 (m, 1 H), 2.28 (s, 3 H), 3.78 (s, 3 H), 3.80−3.91 (m, 2 H), 4.35−4.46 (m, 1 H), 5.11 (s, 2 H), 5.46 (br s, 1 H), 7.30−7.50 (m, 5 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 10.9, 30.8 (t, J = 20 Hz), 46.1 (t, J = 20 Hz), 53.2, 53.4, 67.4, 128.6, 128.8, 129.0; ^{19}F NMR (376 MHz, $CDCl_3$) δ −100.4, −100.7. Anal. ($C_{18}H_{21}N_3O_6F_2$) C, H, N.

***N*^{6}-(Iminoethyl)-5,5-L-difluorolysine Dihydrochloride (32). Step a.** The benzyloxycarbonyl protecting groups were removed as described for compound **10**.

Step b. The methyl ester was removed in refluxing 2 N HCl. After 20 h, the reaction was concentrated under vacuum and subsequently lyophilized. ^{1}H NMR (400 MHz, D_2O) δ 1.99−2.16 (m, 4 H), 2.18 (s, 3 H), 3.73 (t, J = 15 Hz, 2 H), 4.03 (t, J = 5 Hz, 1 H); ^{13}C NMR (100 MHz, D_2O, TSP) δ 19.0, 22.5, 29.9 (t, J = 24 Hz), 46.0 (t, J = 27 Hz), 52.6, 122.2 (t, J = 242 Hz), 167.5, 172.0; ^{19}F NMR (376 MHz, D_2O, $CFCl_3$) δ −105.7. Anal. ($C_8H_{15}N_3O_2F_2$) C, H, N.

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References

(1) Hobbs, A. J.; Higgs, A.; Moncada, S. Inhibition of nitric oxide synthase as a potential therapeutic target. *Annu. Rev. Pharmacol. Toxicol.* **1999**, *39*, 191−220.

(2) Plapp, B. V.; Kim, J. C. Determination of ϵ-acetimidyllysine in proteins. *Anal. Chem.* **1974**, *62*, 291−294.

(3) Moore, W. M.; Webber, R. K.; Jerome, G. M.; Tjoeng, F. S.; Misko, T. P.; Currie, M. G. L-*N*^{6}-(1-iminoethyl)lysine: A selective inhibitor of inducible nitric oxide synthase. *J. Med. Chem.* **1994**, *37*, 3886−88.

(4) Moore, W. M.; Webber, R. K.; Fok, K. F.; Jerome, G. M.; Connor, J. R.; Manning, P. T.; Wyatt, P. S.; Misko, T. P.; Tjoeng, F. S.; Currie, M. G. 2-Iminopiperidine and other 2-iminoazaheterocycles as potent inhibitors of human nitric oxide synthase isoforms. *J. Med. Chem.* **1996**, *39*, 669−672.

(5) Salvemini, D.; Wang, Z. Q.; Wyatt, P. S.; Bourdon, D. M.; Marino, M. H.; Manning, P. T.; Currie, M. G. Nitric oxide: A key mediator in the early and late phase of carrageenan-induced rat paw inflammation. *Br. J. Pharmacol.* **1996**, *118*, 829−38.

(6) Salvemini, D.; Wang, Z. Q.; Bourdon, D. M.; Stern, M. K.; Currie, M. G.; Manning, P. T. Evidence of peroxynitrite involvement in the carageenan-induced rat paw edema. *Eur. J. Pharmacol.* **1996**, *303*, 217−220.

(7) Connor, J. R.; Manning, P. T.; Settle, S. L.; Moore, W. M.; Jerome, G. M.; Webber, R. K.; Tjoeng, F. S.; Currie, M. G. Suppression of Adjuvant-Induced Arthritis by Selective Inhibition of Inducible Nitric Oxide Synthase. *Eur. J. Pharmacol.* **1995**, *273*, 15−24.

(8) Fletcher, D. S.; Widmer, W. R.; Luell, S.; Christen, A.; Orevillo, C.; Shah, S.; Visco, D. Therapeutic administration of a selective inhibitor of nitric oxide synthase does not ameliorate the chronic inflammation and tissue damage associated with adjuvant-induced arthritis in rats. *J. Pharmacol. Exp. Ther.* **1998**, *284*, 714−721.

(9) Hallinan, E. A.; Kramer, S. W.; Houdek, S. C.; Moore, W. M.; Jerome, G. M.; Spangler, D. P.; Stevens, A. M.; Shieh, H. S.; Manning, P. T.; Pitzele, B. S. 4-Fluorinated L-lysine analogues as selective i-NOS inhibitors: Methodology for introducing fluorine into the lysine side chain and references therein. *Org. Biomol. Chem.* **2003**, *1*, 3527−3534.

(10) Furfine, E. S.; Harmon, M. F.; Paith, J. E.; Garvey, E. P. Selective inhibition of constitutive nitric oxide synthase by nitroarginine. *Biochemistry* **1993**, *32*, 8512−8517.

(11) Interchim S. A., Montluçon, France.
(12) Tolman, V.; Beneš, J. Monofluorinated analogues of some aliphatic basic amino acids. *J. Fluorine Chem.* **1976**, *7*, 397−407.
(13) Padron, J. M.; Kokotos, G.; Martin, T.; Markidis, T.; Gibbons, W. A.; Martin, V. S. Enantiospecific synthesis of α-amino acid semialdehydes: A key step for the synthesis of unnatural unsaturated and saturated α-amino acids. *Tetrahedron: Asymmetry* **1998**, *9*, 3381−3394.
(14) Zheng, G−c.; Yoshihara, K.; Kobayashi, Y.; Taguchi, T. Facile synthesis of L-5-fluorolysine from L-homoserine. *Chem. Express* **1990**, *5*, 577−580.
(15) Shirota, F. N.; Nagasawa, H. T.; Elberling, J. A. Potential inhibitors of collagen biosynthesis. 5,5-Difluoro-D,L-lysine and 5,5-dimethyl-D,L-lysine and their activation by lysyl-tRNA ligase. *J. Med. Chem.* **1977**, *20*, 1623−1827.
(16) Kitagawa, O.; Miura, A.; Kobayashi, Y.; Taguchi, T. Atom-transfer reaction of difluoroiodoacetate. *Chem. Lett.* **1990**, 1011−1014.
(17) Fluka, Buchs, Switzerland.
(18) Burton, D. J.; Yang, Z.-Y. A new approach to α,α-difluoro-functionalized esters. *J. Org. Chem.* **1991**, *56*, 5125−5132.
(19) Burton, D. J.; Yang, Z.-Y. Nickel-catalyzed reaction of iododifluoroacetates with alkenes and zinc. *J. Org. Chem.* **1992**, *57*, 5144−5149.
(20) Moormann, A. E.; Wang, J. L.; Palmquist, K. E.; Promo, M. A.; Snyder, J. S.; Scholten, J. A.; Massa, M. A.; Sikorski, J. A.; Webber, R. K. 3-Methyl-4*H*-[1,2,4]-oxadiazol-5-one: A versatile synthon and protecting group for monosubstituted acetamidines. Submitted for publication.
(21) Crane, B. R.; Arvai, A. S.; Ghosh, D. K.; Getzoff, E. D.; Stuehr, D.; Tainer, J. A. Structure of Nitric Oxide Synthases Oxygenase Dimer with Pterin and Substrate. *Science* **1998**, *279*, 2121−2126.
(22) Still, W. C.; Kahn, M.; Mitra, A. Rapid Chromatograhic Technique for Preparative Separation with Moderate Resolution. *J. Org. Chem.* **1978**, *43*, 2923−29.

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