Synthesis and in Vitro Evaluation of ^{18}F- and ^{19}F-Labeled Insulin: A New Radiotracer for PET-based Molecular Imaging Studies

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A new and regioselective strategy was developed for the preparation of fluorine-18-labeled insulin as a novel positron emission tomography (PET) tracer. [^{18}F]-4-Fluorobenzoic acid (4-^{18}FBA), which was produced in 83 ± 8% yield (n = 10), through the use of succinimidyl [^{18}F]-4-fluorobenzoate (4-^{18}FSB), was conjugated through a short spacer (6-aminohexanoic acid, AHx) to the PheB1 residue of a protected form of insulin. ^{18}FB-AHx-insulin (**8b**) was repeatedly prepared in practical quantities (10−20 mCi, 370−740 MBq) in good radiochemical yield (9 ± 5%, n = 9) and in a specific activity of 7.8 mCi/μmol. The final product was characterized by comparing the radioHPLC and radioTLC of **8b** with that of the ^{19}F-analogue (^{19}FB-AHx-insulin, **8a**) and by analyzing a carrier-added synthesis by mass spectrometry. Dithiothreitol and endoproteinase Glu-C digestion experiments on **8a** confirmed that the prosthetic group was in fact conjugated to the PheB1 residue. An insulin receptor (IR) phosphorylation assay using CHO-hIR cells overexpressing recombinant human insulin receptors indicated no statistical difference in the extent of autophosphorylation stimulated by **8a** as compared to that for human insulin (EC_{50} values of 0.82 nM and 1.0 nM, respectively). The stimulation of 2-deoxyglucose uptake in 3T3-L1 mouse adipocytes utilizing **8a** versus unmodified human insulin gave similar EC_{50} values of 0.68 nM and 0.41 nM, respectively. The IC_{50} values for **8a** versus native insulin for the displacement of ^{125}I-insulin from HEK-293 cells were also the same within experimental error (2.6 nM for **8a** versus 2.4 nM for unmodified human insulin). These results support the use of the ^{18}F-insulin analogue as a PET tracer for imaging the distribution of insulin in vivo.

Introduction

The availability of dedicated scanners for imaging small animals is revolutionizing the drug discovery process and the study of biochemical processes.[1,2] Radioimaging is particularly suited for these studies because its sensitivity provides the opportunity to directly visualize the distribution and/or metabolism of specific compounds in living subjects noninvasively. This allows compounds to be studied in a more realistic environment compared to traditional methods, and it also allows longitudinal studies to be performed in the same animals, thereby avoiding subject-to-subject variability.[2] An advantage of using radioimaging over other molecular imaging methods, such as optical imaging, is that the radiotracers can be used for human studies and are not limited to experiments in small animals.

The two main radioimaging modalities are single photon emission computed tomography (SPECT) and positron emission computed tomography (PET). To employ these techniques to evaluate the distribution of drug candidates requires the synthesis and characterization of radiolabeled versions of the compounds under study. Not only must a method be developed to produce the tracer in good yield and purity but nonradioactive analogues must also be prepared and fully characterized. The "cold" compounds are needed to evaluate the impact of introducing the label on the biochemistry of the substrate, which is typically done using in vitro assays. They are also needed as well characterized reference standards for the radiolabeled analogues.

Human insulin is a polypeptide-based hormone involved in the regulation of energy metabolite homeostasis.[3] Specifically, the secretion of insulin in response to high blood glucose levels stimulates the storage of metabolites as glycogen, lipids, and protein for future use when exogenous nutrients are not available.[3] Decreased insulin secretion alone or in combination with peripheral tissue resistance to insulin action leads to hyperglycemia and lipidemia both of which contribute to significantly greater cardiovascular morbidity and mortality in affected individuals.[4] Thus, ameliorating insulin resistance and increasing insulin levels appropriately to restore normal glucose and lipid levels are cornerstones of therapeutic strategies and have resulted in significant improvements in patient's health and quality of life.[5,6] It should also be noted that abnormal increases in insulin utilization are associated with a number of diseases, including diabetes, obesity, hypertension,[4] heart failure,[7] neurodegenerative diseases,[8] and cancer.[9]

A number of radiolabeled analogues of insulin have been reported as tracers for studying the biochemistry of the hormone and its role in disease progression and for evaluating different insulin delivery strategies. ^{125}I-Labeled insulin, in which the isotope is bound to the aromatic ring of A14 tyrosine, is perhaps the best-known radiolabeled insulin derivative, but it is used solely for in vitro studies.[10−14] There are few reports of the synthesis of radiolabeled insulin analogues for in vivo molecular imaging studies using PET. Potential limitations of the studies that have been reported (which involved labeling insulin with ^{18}F[15,16] and ^{124}I[17,18]) include products that are of questionable

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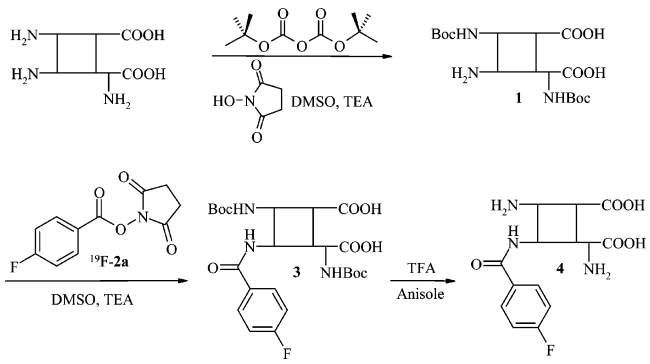
Scheme 1. Preparation of B^1-(4-Fluorobenzoyl)insulin, ^{19}FB-insulin (**4**)



stability, a lack of chemical and biological characterization data, and/or low overall production yields.

Herein a new method for the preparation of ^{18}F-labeled insulin is described. The reported approach overcomes several of the limitations of existing strategies and is accompanied by detailed chemical and in vitro characterization of the nonradioactive (i.e. ^{19}F) analogue. The product of this research can be used to study the distribution and, indirectly, the biochemistry of insulin in vivo using PET.

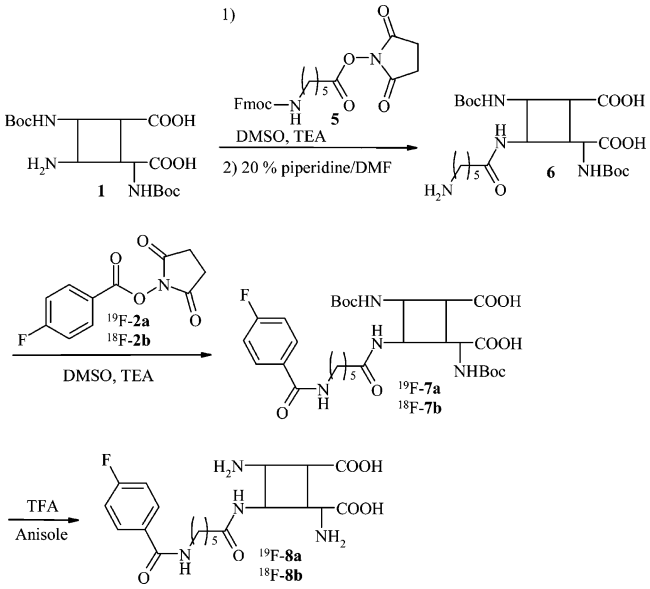
Results and Discussion

Synthesis and Characterization of 4-^{19}FB-AHx-insulin. The first objective was to prepare a ^{19}F-labeled insulin analogue that would be used to determine if the addition of the label would have an impact on the biological activity of the hormone. The approach involved derivatizing the phenylalanine B1 (PheB1) amino acid of insulin with the *N*-hydroxysuccinimidyl active ester of 4-^{19}F-fluorobenzoic acid (4-^{19}FSB). The amine of the PheB1 residue was deemed the most attractive site because this region of the hormone has been shown to not be involved in receptor binding.[19−22] Unfortunately, it has been well-documented that the reactivity of the PheB1 amino residue toward derivatization by electrophiles is less than that of the GlyA1 and LysB29 amino acids.[23] To circumvent this issue, Shai et al. and Baudys et al. developed methods to selectively protect the GlyA1 and LysB29 amines with Boc groups,[15,24] thus providing a suitable precursor (DBI, **1**) for selective derivatization at PheB1.

Compound **1**, which was prepared in 70% yield, was reacted with succinimidyl 4-fluorobenzoate (4-^{19}FSB, **2a**) in the presence of triethylamine (TEA) (Scheme 1). The progress of the reaction between **1** and **2a** was followed by HPLC and it was apparent that conjugation was complete after 20 min. Compound **3** was purified by semipreparative HPLC, and the Boc groups were removed using a mixture of trifluoroacetic acid (TFA)/anisole according to the method described by Lundt et al.[25] Following purification by HPLC, the product (**4**) was lyophilized, yielding a white powder, which showed a single peak in the HPLC (t_R = 12.9 min) and exhibited the expected mass-to-charge ratio in the electrospray mass spectrum (MS) (5928.0 *m*/*z*).

It was later discovered (vide infra) that the PheB1 residue does not react with 4-^{18}FSB when it is prepared in high specific activity. As a result, a short spacer group was attached to the PheB1 residue to increase the reactivity of the amine and to extend the site of conjugation away from the globular structure of insulin. The spacer chain used in this study was 6-aminohexanoic acid (AHx). The amine was protected with an Fmoc group to allow for orthogonal deprotection with respect to the

Scheme 2. Preparation of B^1-([4-Fluorobenzoyl]-6-aminohexanoyl)insulin, FB-AHx-insulin (**8a**, **8b**)



Boc groups on the hormone, while the acid was converted to the active ester to facilitate bioconjugation (Scheme 2). Reaction of **5** with DBI (**1**) was done in a 10:1 molar ratio and was complete in 30 min as shown by HPLC. The resulting conjugate, which was precipitated from solution by the addition of ether, was subsequently treated with 20% (v/v) piperidine in DMF buffer to remove the Fmoc group. The reaction mixture was subsequently passed through a size-exclusion column as a means of removing dibenzofulvene and related derivatives using 100 mM NH_4HCO_3 as the eluent. The purity of the free amine **6** was confirmed by HPLC and the electrospray MS, which showed the expected molecular ion at an *m*/*z* value of 6121.4.

Derivatization of **6** with 4-^{19}FSB was accomplished by adding **2a** to the substrate in DMSO-containing TEA. The reaction was followed by HPLC and the consumption of the amine was completed in 20 min, whereby the MS of **7a** showed a *m*/*z* value of 6243.4, which is consistent with the addition of a single FSB group. The Boc protecting groups were subsequently removed by treatment of **7a** with TFA in the presence of 5% anisole,[25] and the product was isolated by preparative HPLC. After lyophilization, the resulting precipitate was desalted using either dialysis against 100 mM NH_4HCO_3 or size exclusion chromatography using 100 mM NH_4HCO_3 as the eluent. The overall yield of synthesis was approximately 15% and the purity of **8a** was greater than 95% by RP-HPLC.

Liquid chromatography mass spectrometry (LCMS) (electrospray) analysis of **8a** showed a single peak in the HPLC at 6.56 min (Figure 1a), which resulted in three sets of peaks in the mass spectrum at *m*/*z* values of 1209.9, 1512.1, and 2015.4 (calcd 1209.6, 1511.7, and 2015.3, respectively) (Figure 1b). These three sets of peaks correspond to ^{19}FB-AHx-insulin (**8a**) bearing +5, +4, and +3 charges, respectively. Additional peaks in the mass spectrum appeared that correlate with TFA adducts of the parent ion. The MALDI-TOF mass spectrum showed the parent ion at a *m*/*z* value of 6043.8, which is consistent with the calculated mass of **8a**.

The ^{19}F NMR spectrum of **8a** (Figure 2) shows a single multiplet containing broad peaks centered at −109.60 ppm. The $^3J_{FH}$ and $^4J_{FH}$ couplings were 8.9 and 5.6 Hz, respectively. As a reference, the ^{19}F NMR spectra of [^{19}F]-4-fluorobenzoic acid (4-^{19}FBA) and **2a** were acquired, which each show single

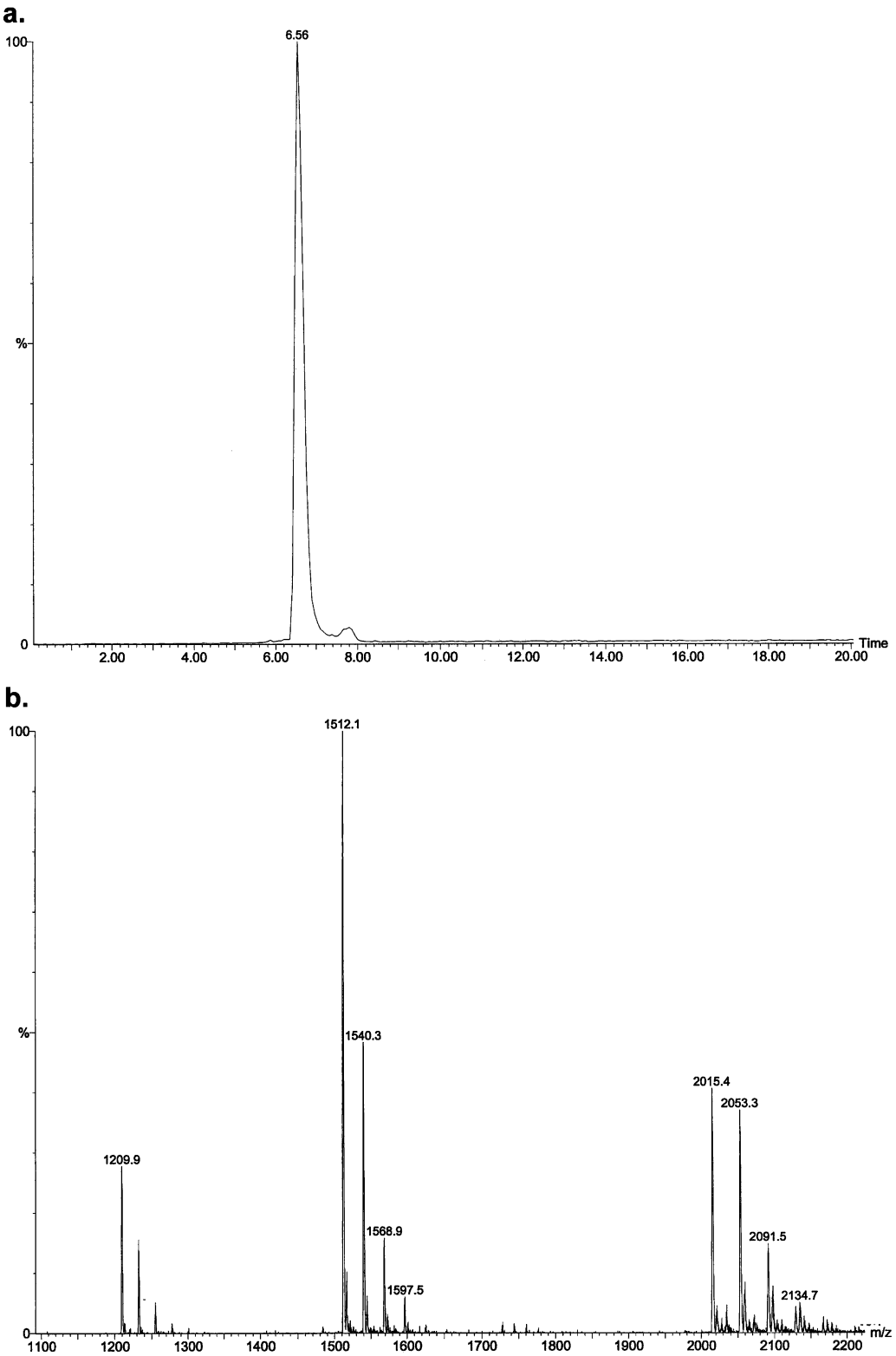


Figure 1. (a) RP-HPLC chromatogram of ^{19}FB-AHx-insulin (**8a**). (b) ES(+) MS of FB-AHx-insulin (**8a**) from LCMS analysis scanned from 1100 to 2200 *m*/*z*.

multiplets (nonets) centered at −106.67 and −101.42 ppm, respectively. The $^{3}J_{FH}$ and $^{4}J_{FH}$ coupling constants for 4-^{19}FBA and compound **2a** are identical to that in **8a**. The broad lines in the ^{19}F NMR spectrum of **8a** and the increased complexity of the observed multiplet are likely the result of the existence of multiple conformations of the hormone derivative, which is consistent with the NMR studies on insulin reported by Weiss et al.[26]

To confirm the site of derivatization, compound **8a** was initially treated with dithiothreitol to disrupt the interchain disulfide bonds.[27] LCMS analysis of the sample (Table 1) showed a peak at 88.36 min with an *m*/*z* value of 1190.1, which corresponds to the molecular ion of the unmodified A-chain. A peak at 104.99 min with an *m*/*z* value of 1222.0 corresponds to the B-chain plus the FB-AHx pendant group attached ([M + H^+]).

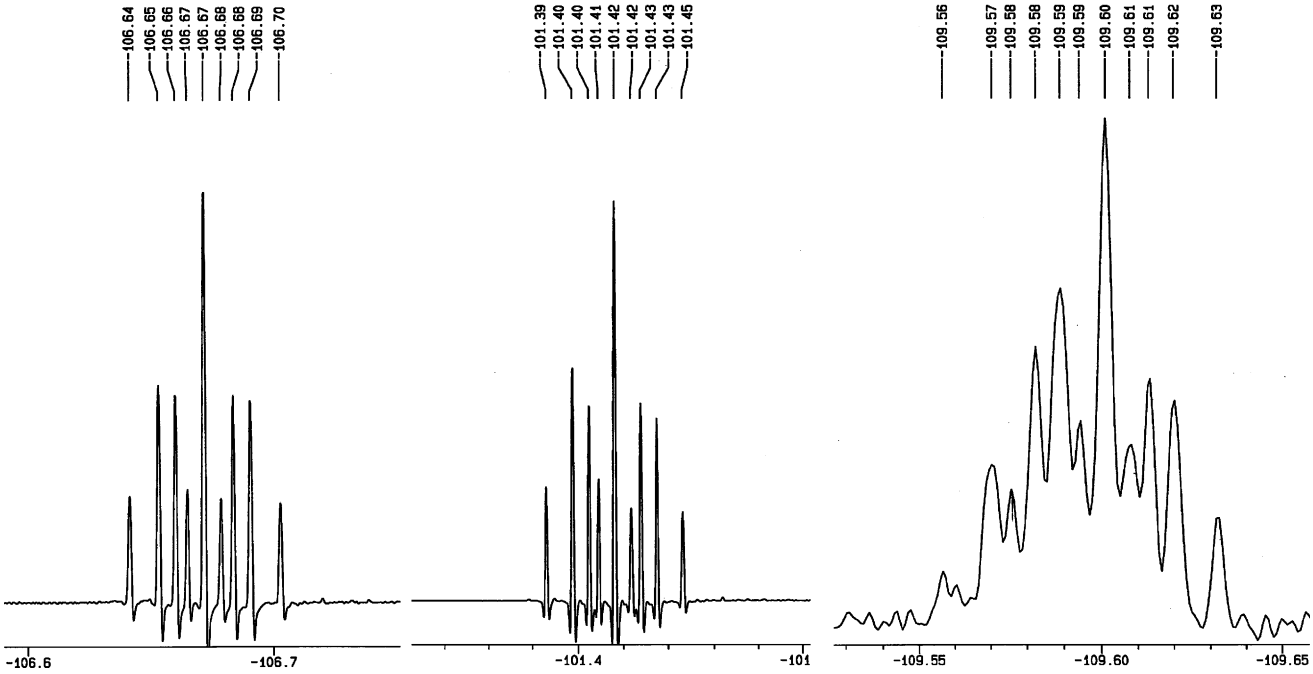


Figure 2. ^{19}F NMR spectra (DMSO-d_6, 470.6 MHz) of 4-fluorobenzoic acid (left), ^{19}FSB (**2a**) (middle), and ^{19}FB-AHx-insulin (**8a**) (right).

Table 1. LCMS (ES+) Studies of ^{19}FB-AHx-insulin (**8a**) Fragments after Dithiothreitol Treatment

HPLC t_R[a]	MS found (calcd)	molecular ion	fragment
88.36	1190.1 (1192.9)	$[M + 2H^+]/2$	A-chain
104.99	1222.0 (1222.8)	$[M + 3H^+]/3$	FB-AHx-B-chain

[a] HPLC column and conditions: C_{18} Beckmann Ultrasphere (150 × 4.6 mm, porosity 5 μm); mobile phase A, H_2O with 0.05% TFA; mobile phase B, 90/10 (v/v) H_2O/CH_3CN with 0.05% TFA; gradient, 99:1 (A/B) for 10 min, 99:1 (A/B) to 11:89 (A/B) for 140 min, 11:89 (A/B) to 99:1 (A/B) for 3 min, 99:1 (A/B) for 17 min; flow rate = 0.1 mL/min.

Table 2. LCMS (ES+) Studies of ^{19}FB-AHx-insulin (**8a**) Fragments after Endoproteinase Glu-C Digestion

HPLC t_R[a]	MS found (calcd)	molecular ion	fragment
65.41	417.5 (417.5)	$[M + H^+]$	GIVE (A-chain)
78.99	1116.6 (1117.3)	$[M + H^+]$	RGFFYTPKT (B-chain)
103.87	1145.1 (1146.3)	$[2M + 3H^+]/3$	FB-AHx-FVNQHLCGSHLVE (B-chain)

[a] HPLC column and conditions: C_{18} Beckmann Ultrasphere (150 × 4.6 mm, porosity 5 μm); mobile phase A, H_2O with 0.05% TFA; mobile phase B, 90/10 (v/v) H_2O/CH_3CN with 0.05% TFA; gradient, 99:1 (A/B) for 10 min, 99:1 (A/B) to 11:89 (A/B) for 140 min, 11:89 (A/B) to 99:1 (A/B) for 3 min, 99:1 (A/B) for 17 min; flow rate = 0.1 mL/min.

The sample was subsequently treated with endoproteinase Glu-C, an enzyme that facilitates cleavage of peptides at the carbonyl side of glutamic acid residues.[27] As demonstrated in Table 2, the peak at 65.41 min resulted in a *m*/*z* of 417.5, which is consistent with the $[M + H^+]$ fragment: GIVE from the A-chain, confirming that derivatization did not occur at the GlyA1 residue. The peak at 78.99 min exhibited an *m*/*z* value of 1116.6, which matches the fragment RGFFYTPKT ($[M + H^+]$) from the B-chain. There was no peak that corresponded to the attachment of the pendant group ^{19}FB-AHx to the lysine residue of RGFFYTPKT from the B-chain. Interestingly, the fragment FB-AHx-FVNQHLCGSHLVE was also not observed; however, the HPLC peak at 103.87 min gave rise to two *m*/*z* values of 859.1 and 1145.1. These correspond to a proton-bound dimer ($[2M + 3H^+]/3$) of the fragment FB-AHx-FVNQHLCGSHLVE, which is consistent with the prosthetic group being attached to the PheB1 residue and not the GlyA1 or LysB29 sites.

In Vitro Screening of 8a. The bioactivity of **8a** was determined in vitro in direct comparison to human insulin using a number of assays. The first assay was an intact cell insulin receptor autophosphorylation assay in CHO cells, which overexpress recombinant human insulin receptors (CHO-hIR). There was no statistically significant difference observed in the extent of autophosphorylation induced by FB-AHx-insulin as compared to unmodified human insulin (Figure 3). The calculated EC_{50} for the autophosphorylation was 0.82 nM for FB-AHx-insulin and 1.0 nM for unmodified human insulin.

The second assay was performed to determine if compound **8a** could stimulate glucose uptake. The extent of 2-deoxyglucose uptake in 3T3-L1 (mouse) adipocyte cells induced by **8a** was not significantly different from that induced by unmodified human insulin (Figure 4). The calculated EC_{50} values were 0.41 nM for human insulin and 0.68 nM for 8a.

The ability of **8a** to bind to the insulin receptor was determined using a displacement assay involving recombinant ^{125}I-insulin and HEK-293 cells expressing the recombinant human insulin receptor. The displacement curves for human insulin and ^{19}FB-AHx-insulin (**8a**) were, within experimental error, equivalent (Figure 4), with 50% displacement achieved at concentrations of 2.6 nM for **8a** and 2.4 nM for unmodified insulin, respectively.

Radiochemistry. ^{18}FB-AHx-insulin (**8b**) was synthesized in fives steps from compound **9** (Schemes 2 and 3), which was prepared following literature procedures.[28] $^{18}F[F^-]$ was added to **9** and Kryptofix222 (4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane) in anhydrous CH_3CN and the mixture heated to 130 °C for 10 min (Scheme 3). Compound **10** was purified using a silica cartridge and was obtained in 76 ± 9% radiochemical yield (n = 10, decay-corrected). TFA was added to compound **10**, cooled in an ice bath, to remove the pentamethylbenzyl-protecting group. After 5 min, the residual TFA was evaporated, which resulted in a significant loss of activity (between 50% and 80%).

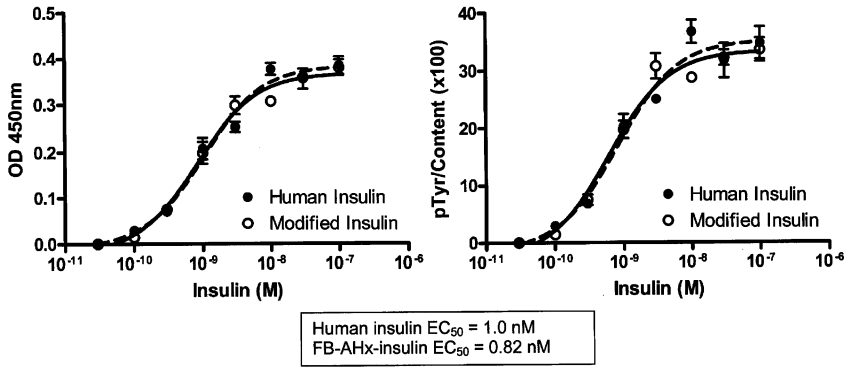


Figure 3. Comparisons of Aventis (human) insulin and ^{19}FB-AHx-insulin (**8a**): insulin receptor autophosphorylation ELISA with 50 mL of CHO-hIR lysate/well (24-well plate); $n = 3$ wells per treatment.

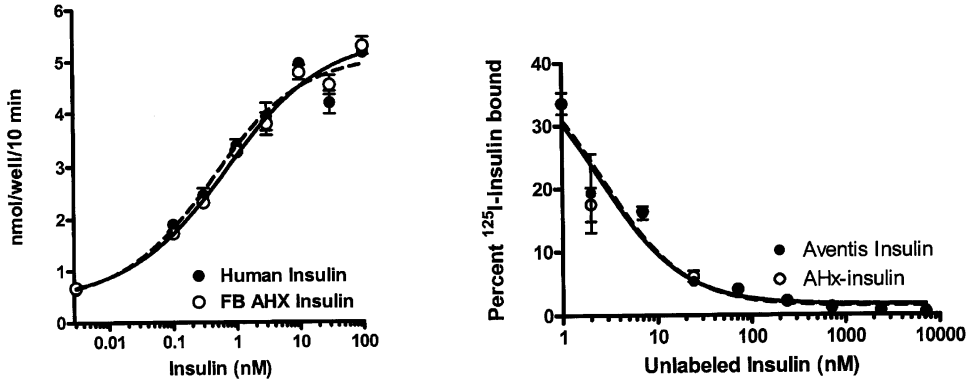
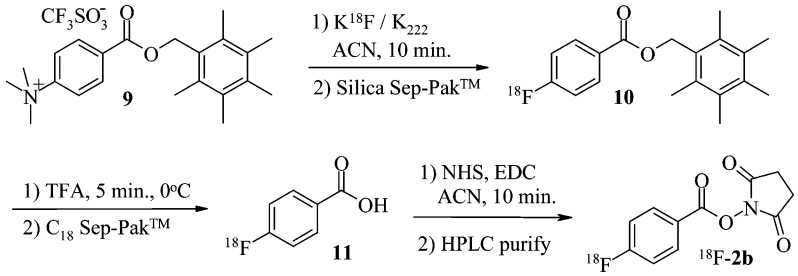


Figure 4. Comparisons of Aventis (human) insulin and ^{19}FB-AHx-insulin (**8a**): insulin dose−response for 2-deoxyglucose uptake in 3T3-L1 adipocytes (left); displacement of bound ^{125}I-insulin in HEK-293 cells expressing recombinant human insulin receptor (right).

Scheme 3. Preparation of Succinimidyl 4-^{18}F-Fluorobenzoate (4-^{18}FSB, ^{18}F-**2b**)



To determine the nature of the volatile species, a trapping experiment was performed where the TFA was removed by blowing N_2 over the sample while passing the gas through a separate vial cooled to −5 °C. The radiochromatogram (HPLC) of the volatile compounds showed that the collected radioactive product was, as expected, compound **11**. The extent of sublimation, even under fairly mild evaporation conditions, was significant.

To alleviate the loss, it is possible to add NMe_4OH to the solution prior to the evaporation step.[29] We found this approach problematic in terms of the base's impact on the reproducibility in the subsequent step involving formation of the active ester. As an alternative approach, following acid deprotection, the resulting mixture was diluted with H_2O (5 mL) and passed through an activated C_{18} cartridge. The cartridge was washed with H_2O (5 mL) to remove any residual TFA and then dried for 1 min using a stream of nitrogen. Compound **11** was subsequently eluted from the cartridge using anhydrous CH_3CN (3 mL) in 88 ± 6% yield ($n = 10$, decay-corrected), and it can be directly converted to the active ester **2b** without having to evaporate the solvent. This approach prevented loss due to sublimation and is easily automated.

To prepare the active ester (Scheme 3), *N*-hydroxysuccinimide and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride in anhydrous CH_3CN were added to the CH_3CN solution of **11**. The reaction was stirred at room temperature for 10 min and the product subsequently isolated by semipreparative HPLC in 83 ± 8% yield ($n = 10$, decay-corrected). HPLC purification of **2b** was necessary to ensure reproducibility in the subsequent conjugation reaction.

Initially, attempts were made to conjugate **2b** to the PheB1 residue of diBoc-insulin **1**, on the basis of the successful synthesis of the nonradioactive analogue. However, formation of the bioconjugate was not observed. Varying the solvent (DMSO, DMF, PBS, or borate buffers), temperature (room temperature to 50 °C), and the amount of **1** (2−18 mg) had no affect on the reaction with the exception of increasing the amount of **11**, which formed as a result of hydrolysis of the active ester.

In contrast to the direct reaction of **2b** with **1**, successful conjugation with **6** was achieved in 20 min (Scheme 2). The product, ^{18}FB-AHx-DBI (**7b**), was isolated from low molecular weight compounds using a size-exclusion cartridge in 43 ± 10% yield ($n = 9$, decay-corrected). The last step involved the

removal of the Boc protecting groups by treating **7b** with neat TFA in the presence of anisole, which was followed by purification using a size-exclusion cartridge. Residual AHx-insulin in the sample can be removed by preparative HPLC (method B, see Experimental Procedures). However, because the effective specific activity was already 7.8 mCi/μmol and because our future imaging studies involve mixing the tracer with a large excess of insulin, further purification was deemed unnecessary. The HPLC retention times of the final product matched that of the ^{19}F reference standard. Typically, 37−74 MBq (1−2 mCi) of B^1-([4-^{18}F-fluorobenzoyl]-6-aminohexanoyl)-insulin (**8b**) was obtained in 180−200 min starting from a 2.78 GBq (75 mCi) of ^{18}F [F$^-$], giving a total yield, based on ^{18}F$^-$, of 9 ± 5% yield (n = 9, decay-corrected). This approach was also amenable to producing significant quantities of the product tracer. Higher production runs starting with 25.2 GBq (680 mCi) yielded 470 MBq (12.7 mCi) of **8b** (decay-corrected yield of 6%).

To further verify that ^{18}FB-AHx-insulin (**8b**) was in fact the product, carrier-added experiments were performed by adding 0.1 mg of ^{19}F$^-$ to the Kryptofix solution prior to the addition of ^{18}F$^-$. The radioactivity was allowed to decay to background and the reaction mixture characterized by MALDI-TOF mass spectrometry. A peak was observed at a m/z value of 6043.8, which is consistent with the mass spectrum of **8a** prepared from **2a**.

Conclusion

A reproducible method for the preparation of a ^{18}F-labeled analogue of insulin was developed. The ^{19}F version was screened in a series of in vitro assays where it was indistinguishable from native insulin. These studies indicate that ^{18}FB-AHx-insulin is a viable PET tracer for studying the distribution and biochemistry of insulin in vivo. The compound is currently being evaluated in a series of PET studies using both small and large animal models, which will be reported in due course.

Experimental Procedures

Materials and Instrumentation. Chemicals were purchased from Aldrich Inc., NovaBiochem Inc., or Fluka Inc. and were used without further purification. Human insulin was obtained from Aventis Inc. Anhydrous CH_3CN (Aldrich, 99.8%), anhydrous DMSO (Aldrich, 99.9%), Kryptofix222 (K_{222}: 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]hexacosane, Aldrich, 98%), $KHCO_3$ (British Drug Houses, 99.5%), and [^{18}O]H_2O (Isotec, 98% ^{18}O) were used where indicated. Saline solution (0.9%, USP grade) was obtained from Baxter Inc. Dibasic sodium phosphate (J. T. Baker, USP grade) solution (0.9%) was prepared immediately prior to use using a sterile container and sterile H_2O designated for human use. Pentamethylbenzyl 4-(*N*,*N*,*N*-trimethylammonium trifluoromethanesulfonate)benzoate (**9**) was synthesized following literature procedures.[28]

TLC was run on plates of silica gel 60-F_{254} (Merck). Nonradioactive compounds were visualized by UV (λ = 254 nm), while radioactive compounds were detected using a BioScan TLC analyzer. Chromatatron plates were made from a slurry of silica gel (60-PF_{254}) containing gypsum (120 g) in deionized H_2O (230 mL) and dried for 24 h at 110 °C.

Size-exclusion chromatography (SEC) was run using Sephadex G-25 resin (Aldrich, Inc.), which was swollen in 100 mM NH_4HCO_3 for 24 h prior to use. The void volume was determined by measuring the volume required to elute a 1 mL solution of Dextran Blue. SEC HiTrap desalting cartridges (GE Healthcare) were activated with 20 mL of 100 mM NH_4HCO_3 (dropwise) immediately prior to use. Following use, the cartridges were washed with 20 mL of buffer, 20 mL of H_2O, and 20 mL of 80/20 (v/v) H_2O/EtOH. Resins were stored in 80/20 (v/v) H_2O/EtOH. Activation of silica cartridges (Waters) was completed immediately prior to use using Et_2O (10 mL), while RP C_{18} cartridges (Waters) were activated with EtOH (10 mL) followed by H_2O (10 mL).

For nonradioactive materials, analytical and semipreparative HPLC experiments were performed using a Varian ProStar system, fitted with a 330 PDA multiwavelength detector, a 230 solvent delivery module, and a Beckmann Ultrasphere C_{18} column (4.6 × 150 mm, 300 Å-5 μm), or a semipreparative Microsorb Dynamax C_{18} column (10 × 250 mm, 300 Å-5 μm). The elution protocol (method A) consisted of a gradient over 20 min from 75/25 A/B (v/v) to 20/80 A/B which was followed by 10/90 A/B for 10 min (mobile phase A = 0.1% TFA in H_2O, mobile phase B = 0.1% TFA in CH_3CN) with the flow rates set at 1.0 and 4.0 mL/min for analytical and semipreparative runs, respectively. The UV detector was set at λ = 254 and 210 nm.

For preparative HPLC runs, a Varian ProStar preparative HPLC system, which consisted of a model 320 uniwavelength detector, a model 215 solvent delivery system, and a Microsorb Dynamax C_{18} column (21.4 × 250 mm, 300 Å-5 μm), was used. The elution protocol (method B) consisted of a gradient from 75/25 A/B (v/v) to 20/80 A/B over 20 min followed by 20/80 A/B (v/v) to 100% B over 5 min (mobile phase A = 0.1% TFA in H_2O, mobile phase B = 0.1% TFA in CH_3CN) at a flow rate of 30 mL/min. The absorbance was monitored at λ = 254 nm.

For the analysis of radioactive compounds, a Hewlett-Packard 1090 analytical system fitted with a diode array multiwavelength detector, a model PV5 solvent delivery system, a NaI detector connected to a Carroll and Ramsey Model 105S ratemeter, and a Beckmann Ultrasphere C_{18} column (4.6 × 150 mm, 300 Å-5 μm) were employed. The elution protocol (method C) consisted of a gradient over 20 min consisting of 75/25 A/B (v/v) to 20/80 A/B and then 10/90 A/B for 10 min (mobile phase A = 0.1% TFA in H_2O, mobile phase B = 0.1% TFA in CH_3CN) at a flow rate of 1.0 mL/min. The absorbance was monitored at λ = 254 nm.

For purification of radioactive materials, a Waters 6000A solvent delivery system fitted with Beckman UV detector (fixed wavelength at 280 nm) and a Bicron GM tube (SWGM B980C) attached to a Bicron ratemeter was employed. Using a semipreparative C_{18} Vydac column (10 × 250 mm, 300 Å-5 μm), the elution protocol (method D) was isocratic 50/50 A/B (v/v) (solvent A = H_2O; solvent B = CH_3CN) at a flow rate of 3.0 mL/min. The absorbance was monitored at λ = 254 nm.

LCMS experiments were run on a Waters 2690 separations module, a model 996 photodiode array multiwavelength detector and a Micromass Quatro Ultima electrospray ionization instrument. For the digestion studies, a C_{18} Beckmann Ultrasphere (150 × 4.6 mm, porosity 5 μm) column was employed using a mobile phase of A: H_2O with 0.05% TFA and B: 90/10 (v/v) H_2O/CH_3CN with 0.05% TFA and a gradient of 99/1 A/B (v/v) for 10 min, 99/1 A/B to 11/89 A/B for 140 min, 11/89 A/B to 99/1 A/B for 3 min and 99/1 A/B for 17 min at a flow rate of 0.1 mL/min.

MALDI-TOF experiments were carried out on a Micromass TOF Spec2E instrument. Prior to each analysis, a calibration standard was run that consisted of a mixture of 1 ρmol/μL substance P, 2 pmol/μL renin substrate tetradecapeptide, 2 pmol/μL adrenocorticotropic hormone fragment 18−39, and 10 pmol/μL cytochrome *c*. This was done in the positive ion reflection mode at 20 kV.

NMR spectra were predominately recorded using a Bruker Avance (AV 200 MHz) instrument and spectra were referenced to the residual proton peaks in deuterated solvents (DMSO-d_6, δ = 2.50 ppm; CD_2Cl_2, δ = 5.32 ppm; CD_3OD, δ = 3.30 and 4.78 ppm) or TMS. Characterization of **8a** was completed using a Bruker AV Ultra Shield Plus (700 MHz) spectrometer at 37 °C. ^{19}F NMR experiments were recorded using a Bruker Avance (DRX 500 MHz) instrument and the chemical shifts referenced to $CFCl_3$. Abbreviations of multiplicity: q = quartet, qu = quintet.

Radiochemistry. Radiosyntheses were performed in a shielded hot cell (7.5 cm lead) using remote manipulators for high level experiments (**Caution**: ^{18}F is radioactive and should be handled in a licensed facility using the appropriate shielding). Specific radioactivity was determined as follows: the area of the absorbance

peak corresponding to the radiolabeled product was measured on the HPLC chromatogram and compared to a standard curve relating mass to absorbance.

Radioisotope Production. No-carrier-added ^{18}F-fluoride was produced using a Siemens 11 MeV proton-only cyclotron (RDS 112) using an $^{18}O(p,n)^{18}F$ reaction. The $[^{18}O]H_2O$ was separated from $^{18}F^-$ by passing the mixture through an anion exchange column (BioRad, AG 11 A8 resin, 50−500 mesh, converted to HCO_3^- form). The $^{18}F^-$ was subsequently eluted from the column using of 95/5 (v/v) CH_3CN/H_2O (1 mL) solution containing 8 mg of K_{222} and 2 mg $KHCO_3$ and collected in a 10-mL vial, and the solvent was evaporated at 130 °C. The residue was redissolved in anhydrous CH_3CN (0.5 mL) and evaporated to dryness. The drying procedure was repeated a second time before use.

Synthetic Procedures. A^1,B^{29}-Di(*tert*-butyloxycarbonyl)insulin (DBI, 1). A modification of the literature procedure reported by Shai et al. was employed.[15] A solution of di-*tert*-butyl dicarbonate (0.188 g, 0.86 mmol), TEA (200 μL), and *N*-hydroxysuccinimide (0.207 g, 1.80 mmol) was stirred in DMSO (500 μL) for 1 h. The solution was subsequently added dropwise over 2 h (20 gauge needle, 1 drop every 30 s) via a 1-mL syringe to a rapidly stirring solution of human insulin (2.0 g, 0.344 mmol) dissolved in DMSO (40 mL) containing 5% TEA (v/v). The reaction mixture was allowed to stir for 1 h and subsequently divided equally among eight centrifuge vials, which contained 30 mL of cold Et_2O. Precipitation was induced through the addition of 5−8 mL CH_3CN. The solid was isolated by centrifugation (3200 rpm) at 5 °C. The residue was washed twice with 50/50 (v/v) Et_2O/CH_3CN and isolated by centrifugation. The solid was dissolved in a 75/25 (v/v) H_2O/CH_3CN containing 0.1% TFA and the desired product purified using preparative HPLC (method B). Fractions collected were concentrated by rotary evaporation (water bath at 30°C) to remove the majority of the CH_3CN followed by lyophilization overnight, which yielded a white solid. The solid was subsequently desalted by SEC using a 100 mM NH_4HCO_3 buffer as the eluent. Following lyophilization, compound **1** (1.49 g, 72%) was obtained as a colorless solid. Anal. HPLC (method A): t_R = 7.80 min. MS *m*/*z* ES(+): found, 1202.4 [M + 5H$^+$]/5, 1503.0 [M + 4H$^+$]/4, 2004.0 [M + 3H$^+$]/3; calcd, 1202.8, 1503.0, 2003.6, respectively.

Succinimidyl 9-Fluorenylmethoxycarbonyl-6-aminohexanoate (5). To a solution of 9-fluorenylmethoxycarbonyl-6-aminohexanoic acid (1.0 g, 2.83 mmol) in CH_3CN (500 mL) were added *N*-hydroxysuccinimide (0.411 g, 3.57 mmol) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.698 g, 3.57 mmol). The reaction was allowed to stir at room temperature for 2 h, at which time it was concentrated to dryness using a rotary evaporator. The residue was dissolved in CH_2Cl_2 (25 mL) and extracted with H_2O (2 × 20 mL). The organic layer was dried with $MgSO_4$, filtered, and then concentrated to dryness using a rotary evaporator. The residue was redissolved in CH_2Cl_2 (1−2 mL) and purified using a chromatotron and a solvent gradient consisting of 100% CH_2Cl_2 to 90/10 (v/v) CH_2Cl_2/CH_3OH. The product (0.83 g, 65%) was a colorless solid. R_f (DCM/MeOH 95/5, v/v): 0.78. Mp = 110−113 °C. ^{1}H NMR (CD_2Cl_2): δ 7.78 (d, 2H, J = 7.5 Hz); 7.62 (d, 2H, J = 7.4 Hz); 7.44 (dd, 2H, J = 7.5, 7.4 Hz); 7.32 (dd, 2H, J = 7.4, 7.4 Hz); 4.98 (br s, 1H); 4.37 (d, 2H, J = 6.7 Hz); 4.22 (t, 1H, J = 6.5 Hz); 3.17 (d, 2H, J = 6.2 Hz); 2.79 (s, 4H); 2.61 (t, 2H, J = 7.2 Hz); 1.76 (qu, 2H); 1.53 (qu, 2H); 1.44 (m, 4H). ^{13}C NMR (CD_2Cl_2): δ 169.87, 169.24, 156.80, 144.77, 141.85, 128.16, 127.55, 125.64, 120.45, 66.83, 47.93, 41.15, 31.39, 29.89, 26.26, 26.19, 24.81. IR (KBr, cm^{-1}): ν 3366.10 (N−H), 1725.87 (C═O), 1693.77 (C═O), 1528.69 (C═C). MS *m*/*z* ES(+): found, 451.3 [M + H$^+$]; calcd, 451.5.

A^1,B^{29}-Di(*tert*-butyloxycarbonyl)-B^1-(6-aminohexanoyl)insulin (AHx-DBI, 6). A solution of DBI (**1**) (40.7 mg, 6.77 μmol) and **5** (30.5 mg, 67.7 μmol) in DMSO (400 μL) containing 5% TEA (v/v) was allowed to stir at room temperature for 30 min. The reaction mixture was transferred to a centrifuge vial containing cold Et_2O (2 mL) and precipitation induced by the addition of CH_3CN (1.5 mL). The resulting solid was isolated by centrifugation (3200 rpm) at 5 °C, washed twice with a 50/50 (v/v) Et_2O/CH_3CN solution, and reisolated by centrifugation. The residue was collected by filtration through a fritted funnel. Anal. HPLC (method A): t_R = 12.2 min. MS *m*/*z* ES(+): found, 1586.8 [M + 4H$^+$]/4, 2116.2 [M + 3H$^+$]/3; calcd, 1586.9, 2115.5, respectively.

The precipitate was transferred to a vial and DMF (600 μL) containing 20% piperidine (v/v) added. The reaction was allowed to stir for 20 min, at which time the mixture was transferred to a centrifuge vial containing cold Et_2O (2 mL) and precipitation induced by the addition of CH_3CN (1.5 mL). Isolation of the solid followed the same procedure described above. The solid was dissolved in 75/25 (v/v) H_2O/CH_3CN containing 0.1% TFA and the desired product purified using preparative HPLC (method B). The collected fractions were concentrated by rotary evaporation (water bath at 30°C) to remove the majority of the CH_3CN followed by lyophilization overnight, which yielded a white solid. The solid was subsequently desalted by SEC using a 100 mM NH_4HCO_3 buffer as the eluent. Following lyophilization, compound **6** (29 mg, 69%) was obtained as a colorless solid. Anal. HPLC (method A): t_R = 8.91 min. MS *m*/*z* ES(+): found, 1531.1 [M + 4H$^+$]/4, 2041.7 [M + 3H$^+$]/3; calcd, 1531.3, 2041.3, respectively.

A^1,B^{29}-Di(*tert*-butyloxycarbonyl)-B^1-([4-fluorobenzoyl]-6-aminohexanoyl)insulin (^{19}FB-AHx-DBI, 7). A solution of **6** (10.0 mg, 1.63 μmol) and succinimidyl 4-fluorobenzoate (**2a**) (3.87 mg, 16.3 μmol) in DMSO (100 μL) containing 5% TEA (v/v) was allowed to stir at room temperature for 20 min. The reaction mixture was transferred to a centrifuge vial containing cold Et_2O (1 mL), whereupon precipitation was induced by the addition of CH_3CN (0.5 mL). Isolation of the solid was accomplished by centrifugation at 5 °C (3200 rpm). Compound **6** was isolated by semipreparative HPLC (method A) which, after lyophilization, resulted in a colorless solid (5.7 mg, 56%). Anal. HPLC (method A): t_R = 11.4 min. MS *m*/*z* ES(+): found, 1561.6 [M + 4H$^+$]/4, 2082.7 [M + 3H$^+$]/3; calcd, 1562.0, 2082.4, respectively.

B^1-([4-Fluorobenzoyl]-6-aminohexanoyl)insulin (^{19}FB-AHx-insulin, 8a). To a vial containing **7** (2.1 mg, 0.34 μmol) were added anisole (5 μL, 46.0 μmol) and TFA (100 μL), and the reaction was allowed to stir at room temperature for 10 min. The reaction mixture was transferred to a centrifuge vial containing cold Et_2O (1 mL) and the resulting precipitate isolated by centrifugation at 5 °C (3200 rpm). The target compound was isolated by semipreparative HPLC (method A), the organic solvent removed by gentle rotary evaporation, and the resulting solution lyophilized overnight. The solid residue was desalted using SEC and a 100 mM NH_4HCO_3 buffer, producing a colorless solid (1.5 mg, 74%) after lyophilization. ^{19}F NMR (DMSO-d_6): δ −109.60 (tt, 1F, J = 8.9, 5.6 Hz); −109.58 (t, J = 8.9 Hz). Anal. HPLC (method A): t_R = 9.15 min. MS *m*/*z* ES(+): found, 1209.9 [M + 5H$^+$]/5, 1512.1 [M + 4H$^+$]/4, 2015.4 [M + 3H$^+$]/3; calcd, 1209.6, 1511.7, 2015.3, respectively.

Digestion Studies. A total of 25 μL of 0.4 M NH_4HCO_3(aq) was added to 250 μL of **8a** (2.4 mg/mL) in PBS in a 1.5 mL conical vial and the mixture agitated gently. To this was added 5 μL of 45 mM aqueous dithiothreitol and the mixture incubated for 15 min at 50 °C. After cooling to room temperature, 5 μL of 100 mM aqueous iodoacetamide was added and the solution agitated periodically over 15 min. An aliquot (56 μL) was mixed with 28 μL of 0.4 M NH_4HCO_3 in 8 M aqueous urea and the analysis performed by LCMS (ES+) over 160 min.

The remaining solution from the dithiothreitol experiment was transferred to a glass vial. To this was added 2.5 μL of endoproteinase-Glu-C in MilliQ water and the mixture incubated for 16 h at 37 °C. An aliquot (56 μL) was mixed with 28 μL of 0.4 M NH_4HCO_3 in 8 M aqueous urea. Analysis was preformed by LCMS (ES+).

Receptor Binding Assay. A human embryonic kidney (HEK-293) cell line that that has been stably transfected to express human insulin receptors was incubated in the presence of 1.4 × 10^{-10} M of ^{125}I-insulin and varying concentrations of unlabeled human insulin or FB-AHx-insulin. Cells were incubated in 50 mM PBS with 0.5% BSA for 120 min at 4 °C and then washed three times. The pelleted cells were counted on a γ-counter and results were reported as percent binding (cpm sample/cpm added × 100).

For cellular autophosphorylation assays, CHO-hIR cells were grown in α-MEM, 10% FBS with 10 μg/mL gentamicin, serum deprived for 1 h, and then incubated with either human insulin (Aventis) or FB-AHx-insulin at the indicated concentrations for 10 min. Cells were then lysed in a buffer consisting of 50 mM HEPES, pH 7.4, 150 mM NaCl, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 0.1 mM EGTA, and 1 Roche EDTA-free Protease Inhibitor Cocktail Tablet (per 50 mL volume). Lysates were clarified by centrifugation at 100 000*g* for 5 min at 4 °C and applied to 96-well plates coated with anti-insulin receptor monoclonal antibodies (29B4 antibody). The extent of autophosphorylation was quantified using a second antibody directed against phosphotyrosine residues (HRP-conjugated PY20, Oncogene Research Products) and a coupled horseradish peroxidase reaction (ELISA). 2-Deoxyglucose uptake in 3T3-L1 adipocytes in response to human insulin or FB-AHx-insulin at the indicated concentrations was determined as previously described.[30]

Radiosynthesis. B^1-([4-^{18}F-Fluorobenzoyl]-6-aminohexanoyl)-insulin (^{18}FB-AHx-insulin, ^{18}F-8b). To an anhydrous CH_3CN (500 μL) solution of ^{18}F-fluoride containing 8 mg of K_{222} was added pentamethylbenzyl 4-(*N*,*N*,*N*-trimethylammonium trifluoromethanesulfonate)benzoate (**9**)[28] (3.0 mg, 6.13 μmol) dissolved in anhydrous CH_3CN (500 μL). The vial was capped tightly and placed in a heating block at 130 °C for 10 min with stirring. The reaction vessel was cooled using an ice−water bath and the activity measured. The resulting light yellow solution was diluted with Et_2O (3 mL) and passed through an activated silica cartridge collecting ^{18}F-**10** in a 10-mL vial. The cartridge was washed with Et_2O (3 mL) and the activity combined with the initial eluent to give the desired product in 67−81% yield. Anal. HPLC (method C): t_R = 25.7 min. SiO_2-TLC: CH_3CN/H_2O 95/5 (v/v), R_f = 0.97.

The Et_2O solvent was removed by heating the solution to 90 °C while passing a gentle stream of nitrogen over the reaction vial for 4−6 min. The dried sample was subsequently placed in a water-ice bath, neat TFA (200 μL) added, and the mixture stirred for 5 min. The solution was diluted with H_2O (5 mL) and passed through an activated C_{18} cartridge. The cartridge was washed with H_2O (5 mL) and then dried for 1 min by applying a stream of nitrogen. 4-^{18}F-Fluorobenzoic acid (^{18}FBA) (^{18}F-**11**) was eluted from the Sep-Pak using anhydrous CH_3CN (3 mL), which was collected in a 10-mL vial in 77−95% yield. Anal. HPLC (method C): t_R = 7.89 min. SiO_2-TLC: CH_3CN/H_2O 95/5 (v/v), R_f = 0.34.

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (34 mg, 0.17 mmol) and *N*-hydroxysuccinimide (20 mg, 0.17 mmol) was added to **11** in anhydrous CH_3CN (500 μL). The reaction was stirred at room temperature for 10 min, whereupon it was concentrated to approximately a 0.5−1 mL volume by heating the solution at 90 °C for 6−8 min under a gentle stream of nitrogen. To this sample was added enough H_2O to make a total volume of 1.5 mL. The solution was purified by semipreparative HPLC (method D), collecting the peak corresponding to the succinimidyl 4-^{18}F-fluorobenzoate (4-^{18}FSB). The HPLC eluent was transferred to a 20-mL syringe containing 14 mL of H_2O, and the entire volume was passed through an activated C_{18} cartridge. After drying the cartridge under a stream of nitrogen (1 min), pure **2b** was collected (71−91% yield) in a 10-mL vial by flushing CH_3CN (3 mL) through the cartridge. Anal. HPLC (method C): t_R = 10.53 min. SiO_2-TLC: CH_3CN/H_2O 95/5 (v/v), R_f = 0.88.

The solution containing **2b** was concentrated to dryness at 90 °C under a slow stream of nitrogen (6−8 min). To the dried sample was added **6** (2 mg, 0.16 μmol) dissolved in DMSO (200 μL) containing 10% TEA (v/v). The reaction was allowed to stir at room temperature for 20 min and subsequently diluted with 100 mM NH_4HCO_3 (1 mL). The reaction mixture was loaded onto an activated SEC Sep-Pak, which was washed with 2 mL of 100 mM NH_4HCO_3. The product was subsequently eluted using an additional 3 mL of 100 mM NH_4HCO_3, which was collected in a 10-mL vial. This fraction was loaded onto a C_{18} cartridge, which was washed with H_2O (5 mL) and dried for 1 min using a stream of nitrogen. The desired product was isolated in 32−53% yield by passing CH_3CN (3 mL) containing 0.1% TFA (v/v) through the cartridge. Anal. HPLC (method C): t_R = 12.1 min.

The CH_3CN solution of **7b** was concentrated to dryness by passing a stream of nitrogen over the sample, which was heated to 30 °C for approximately 15−20 min. TFA (400 μL) containing 5% anisole (v/v) was then added and the mixture allowed to stir for 5 min, at which time, the TFA was removed under a stream of nitrogen (2−3 min). The residue was redissolved in EtOH (200 μL) and 50/50 $NaCl/Na_2HPO_4$ (800 μL, 0.9%, USP grade). The mixture was transferred to an activated SEC cartridge, which was eluted with 2 mL 50/50 $NaCl/Na_2HPO_4$ (0.9%, USP grade), and the 3 mL of buffer was collected in one vial. The solution containing **8b** (31−77% yield) was filtered through a Millipore frit prior to use. Anal. HPLC (method C): t_R = 10.7 min.

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Supporting Information Available: HPLC data and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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