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One Step Radiosynthesis of 6-[^{18}F]Fluoronicotinic Acid 2,3,5,6-Tetrafluorophenyl Ester ([^{18}F]F-Py-TFP): A New Prosthetic Group for Efficient Labeling of Biomolecules with Fluorine-18

Dag E. Olberg,*,† Joseph M. Arukwe,‡ David Grace,‡ Ole K. Hjelstuen,†,‡ Magne Solbakken,‡ Grete M. Kindberg,‡ and Alan Cuthbertson‡

†Department of Pharmaceutics and Biopharmaceutics, Institute of Pharmacy, University of Tromsø, N-9037 Tromsø, Norway and ‡GE Healthcare MDx R&D, Nycoveien 2, NO-0401 Oslo, Norway

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The labeling of biomolecules for positron emission tomography (PET) with no-carrier-added fluorine-18 is almost exclusively accomplished using prosthetic groups in a two step procedure. The inherent complexity of the process renders full automation a challenge and leads to protracted synthesis times. Here we describe a new ^{18}F-labeled prosthetic group based on nicotinic acid tetrafluorophenyl ester. Reaction of [^{18}F]fluoride at 40 °C with the trimethylammonium precursor afforded 6-[^{18}F]fluoronicotinic acid tetrafluorophenyl ester ([^{18}F]F-Py-TFP) directly in 60−70% yield. [^{18}F]F-Py-TFP was conveniently purified by Sep-Pak cartridge prior to incubation with a peptide containing the RGD sequence. The desired conjugate was formed rapidly and in good yields. An in vitro receptor-binding assay for the integrin $\alpha_v\beta_3$ was established to explore competition with peptide and peptidomimetic prepared from F-Py-TFP with ^{125}I-echistatin. The nonradioactive conjugates were found to possess high binding affinities with calculated K_i values in the low nanomolar range.

Introduction

Fluorine-18 is among the radionuclides for positron emission tomography (PET[a]) having the most suitable characteristics for in vivo imaging. This is due to a relatively long half-life (109.7 min), high positron abundance (97%), and low positron emission energy (max 635 keV) combined with a well established production method allowing for multi-Curie batches through the ^{18}O(p,n)^{18}F reaction.[1−4]

Currently, 2-[^{18}F]fluoro-2-deoxy-D-glucose (FDG) is by far the most widely available PET tracer supported in part by the cost-effective, high yielding automated production methods.[5,6] There are, however, many other biomolecules that show promise for molecular imaging with PET where efficient fluorine-18 incorporation still represents a significant challenge to the radiochemist. Peptide-based ^{18}F-tracers have already demonstrated clinical utility for imaging various types of diseases, the most important being in the field of oncology.[7,8] Complex peptides can be readily synthesized using solid-phase chemistry and can be tailored to target the desired tissue/receptor with high specificity.[9,10] In addition, they display fast excretion kinetics, making them very suitable for in vivo imaging.[11,12] Examples include ^{18}F-conjugates of Arg-Gly-Asp (RGD) and bombesin peptides, both molecular classes having shown promise for the noninvasive detection of tumors with PET.[13−16]

The automated radiosynthesis of peptide-based PET radiopharmaceuticals remains the Achilles' heel of PET radiopharmaceutical manufacture. High yielding reactions for incorporation of fluorine-18 into complex biomolecules are essential for successful commercial adoption because of the high cost of goods of both peptide and fluoride production.

The labeling of peptides with [^{18}F]fluoride is most commonly performed by reaction of an ^{18}F-prosthetic group with a suitable fuctionalized peptide vector. An example of such a prosthetic group is *N*-succinimidyl 4-[^{18}F]fluorobenzoate ([^{18}F]SFB); its radiosynthesis requires two to three steps and HPLC purification prior to conjugation with peptide.[17−19] Furthermore, following conjugation with [^{18}F]SFB an additional purification step is often required to obtain the ^{18}F-peptide with sufficient specific activity and purity for in vivo studies. This complex multistep radiosynthesis complicates the route to automated production of the final ^{18}F-peptide drug product. From a radiochemical standpoint the key challenge is therefore to develop new methodologies that simplify the manufacturing process.

Attempts to directly label active ester based prosthetic groups with ^{18}F have been previously reported in the literature; however, degradation of the active ester under the harsh ^{18}F-labeling conditions resulted in disappointingly low radiochemical yield.[20,21] We hypothesized that active esters of nicotinic acid functionalized with a suitable leaving group at the 2-position would be highly activated for nucleophilic aromatic substitution with [^{18}F]fluoride and allow direct labeling under conditions where the active ester moiety would be less prone to degradation. The 2-[^{18}F]fluoropyridines can be produced in high yields with high specific activity and have

*To whom correspondence should be addressed. Phone: +47 41517119. Fax: +47 23186014. E-mail: dag.erlend.olberg@petsenteret.no.

[a] Abbreviations: DCC, *N,N'*-dicyclohexylcarbodiimide; K222, kryptofix 222; PEG, polyethylene glycol; PET, positron emission tomography; RCP, radiochemical purity; RGD, Arg-Gly-Asp; t_R, retention time; [^{18}F]SFB, *N*-succinimidyl 4-[^{18}F]fluorobenzoate; TBA, tetrabutylammonium; *t*-BuOH, 2-methyl-2-propanol; TFP-OH, 2,3,5,6-tetrafluorophenol; TMSOTf, trimethylsilyl trifluoromethanesulfonate.

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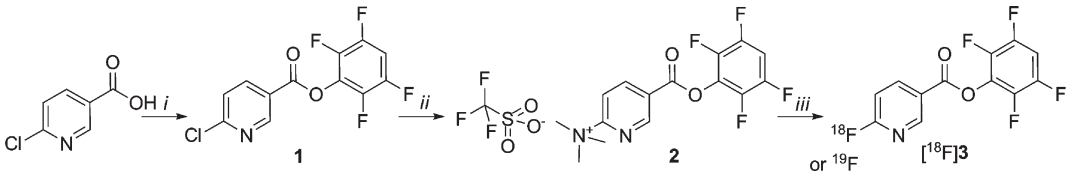
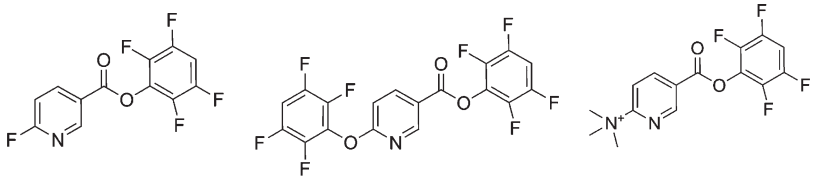
Scheme 1. Synthesis of Precursor **2** and Prosthetic Group [^{18}F]**3** or Nonradioactive **3**[a]



[a] Reagents and conditions: (i) TFP-OH, DCC, DCM, overnight; (ii) (1) trimethylamine, THF, 3 h; (2) TMSOTf, DCM; (iii) KF, K222, MeCN, room temperature, 15 min (nonradioactive **3**) or TBA-[^{18}F], *t*-BuOH/MeCN (8:2), 40 °C, 10 min.

Table 1. Summary of the Main Reaction Components As Identified in NMR Experiments at Different Time Points[a]



3 (F-Py-TFP) **4** **2**

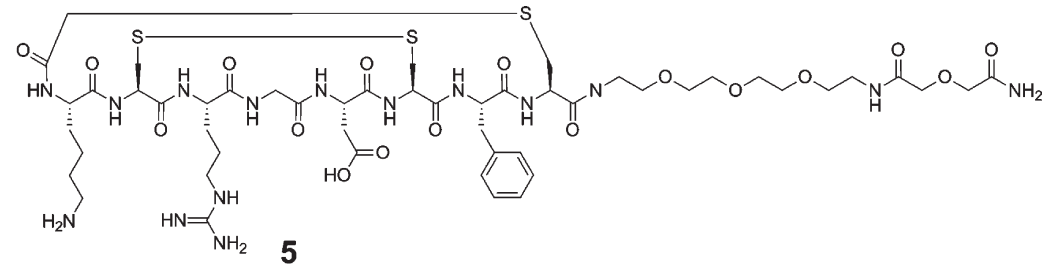
Time (min)	RATIO		
5	63	11	26
10	70	15	15
12	71	16	13
15	72	16	12

[a] The ratios are derived from the mole ratio from the ^{1}H NMR spectra.

the advantage that they do not require an additional electron-withdrawing substituent for activation, as is common for the nucleophilic homoaromatic substitutions.[22−24] Furthermore, they are known to be stable in vivo and therefore represent a plausible alternative to [^{18}F]fluorohomoaromatic systems.[25−27] Herein, we describe the synthesis and application of a novel prosthetic group based on nicotinic acid tetrafluorophenyl (TFP) ester. A single radiosynthesis step is required, affording 6-[^{18}F]fluoronicotinic acid tetrafluorophenyl ester ([^{18}F]F-Py-TFP, [^{18}F]**3**) in good yields from the trimethylammonium precursor. The resulting prosthetic group is readily purified on a Sep-Pak cartridge prior to conjugation with the peptide precursor. By use of this methodology, an ^{18}F-RGD peptide was synthesized in 20−30% isolated yield (decay corrected) based on [^{18}F]fluoride within 90 min.

Results and Discussion

Chemistry. The synthesis of precursor **2** from 6-chloronicotinic acid and its fluorination by nucleophilic aromatic substitution affording nonradioactive **3** are outlined in Scheme 1. Precursor **2** was obtained in good yields from 6-chloronicotinic acid in three steps. Ester formation using *N*,*N*′-dicyclohexylcarbodiimide (DCC) of the starting material with 2,3,5,6-tetrafluorophenol (TFP-OH) produced intermediate **1** which was readily purified by crystallization from hexane in good yield. Normally trimethylammonium precursors are obtained by methylation of substituted dimethylanilines with strong methylating agents such as methyl trifluoromethanesulfonate.[28] In this case we exploited the fact that the chloro-substituent of intermediate **1** was highly activated toward nucleophilic aromatic substitution and further enhancement was provided through the electron-withdrawing tetrafluorophenyl ester moiety. By use of conditions similar to those described by Barlin et al.,[29] the 2-trimethylammonium chloride salt of nicotinic acid TFP ester could be conveniently obtained in high purity as a precipitate from the reaction of the chloride **1** with trimethylamine in THF. Because of the poor solubility of the chloride salt of **2** in acetonitrile, the counterion was changed to the trifluoromethanesulfonate salt before progressing further. Addition of silver triflate in acetonitrile to the chloride salt gave target compound **2**; however, this method required chromatographic purification of the product. More conveniently, addition of trimethylsilyl trifluoromethanesulfonate (TMSOTf) to a suspension of the chloride salt in dichloromethane afforded **2** directly following filtration of solid material and evaporation in vacuo of the organic phase. The residue was precipitated and washed well with diethyl ether to obtain pure **2**. This triflate salt provided an inert counterion along with excellent solubility of the precursor in acetonitrile. It was noteworthy that attempts to synthesize an *N*-hydroxysuccinimide (NHS) ester analogue proved unsuccessful, as extensive decomposition of the ester occurred on contact with trimethylamine (data not shown). The precursor **2** was progressed further to investigate its reaction with fluoride ion. By employment of a standard fluorination protocol with potassium fluoride/kryptofix 222 (K222) complex in acetonitrile at room temperature (15 min), a white precipitate was observed in the reaction mixture along with a distinct odor of trimethylamine. LC−MS analysis of the reaction mixture revealed a product eluting in the void volume which was identified as products from precursor



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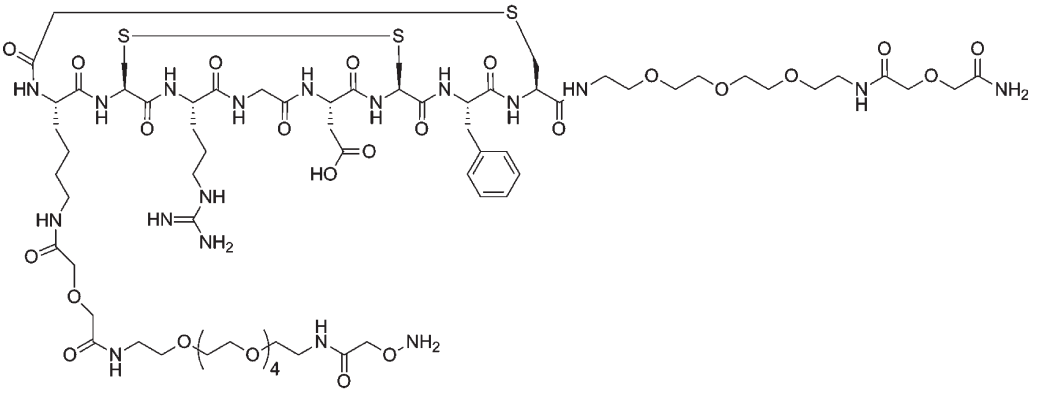

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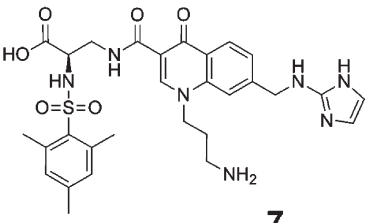

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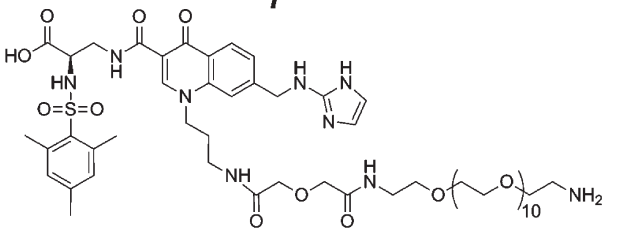

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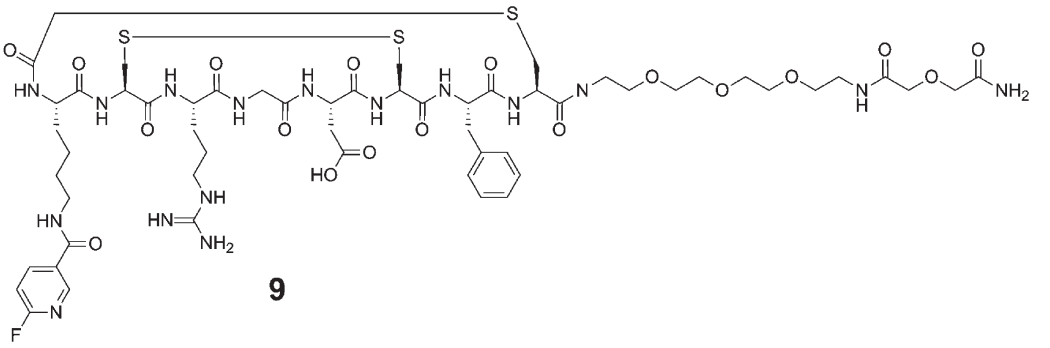

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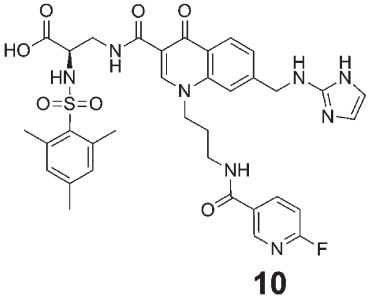

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Figure 1. (Continued on next page).

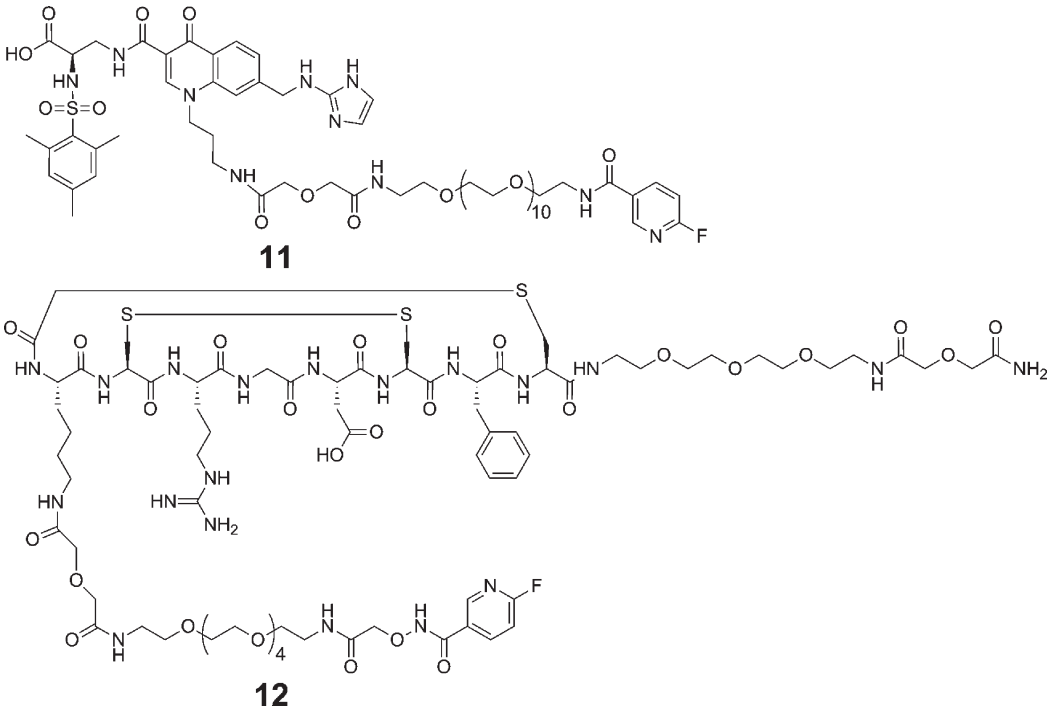

Figure 1. Structures of peptides and peptidomimetics with their corresponding conjugates.

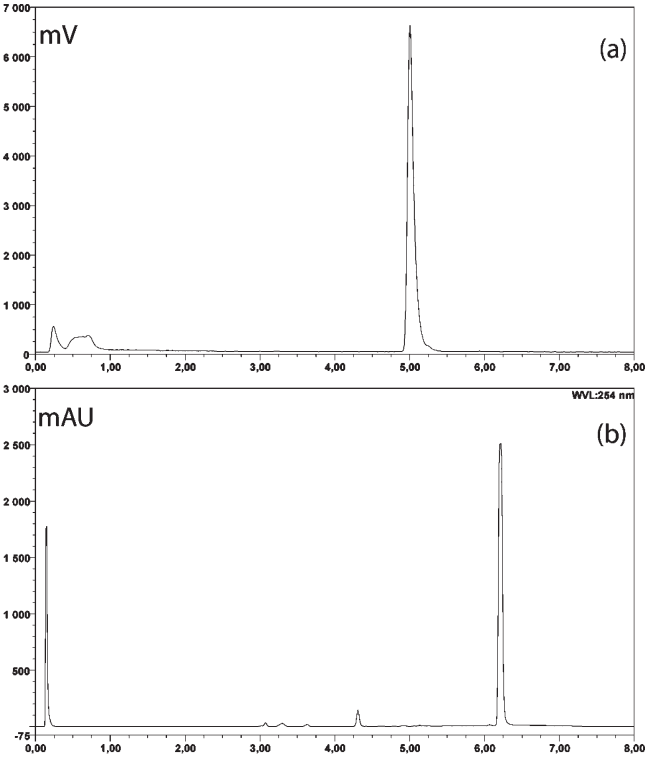

Figure 2. Radio-HPLC (system 1) analysis of an aliquot taken from the crude reaction using TBA-HCO_3/*t*-BuOH/MeCN system after 10 min at 40 °C: (a) radioactive channel ([^{18}F]**3** = 5.0 min peak); (b) UV channel at 254 nm (**4** = 6.2 min peak).

hydrolysis. A small peak corresponding to starting material **2** was also identified with a retention time of 1.75 min, but interestingly two major new products eluting at 3.54 and 4.81 min in the chromatogram were observed; however, LC−MS was unable to resolve the identity of these main products. Following purification of the reaction mixture by normal-phase chromatography, the two products were isolated for their identification by NMR. In addition, an in-depth NMR study of the fluorination reaction was performed by immediate addition of precursor **2** to the KF/K222 complex in MeCN-d_3. The progress of the reaction was followed for approximately 25 min acquiring multiple ^{19}F- and ^{1}H-spectra. NMR spectra of the two major products confirmed the structure of the 3.54 min peak to be target compound **3**. Interestingly, the 4.81 min peak proved to be a side product of nicotinic acid TFP ester where the trimethylammonium moiety of precursor **2** was substituted with a TFP ether as shown in Table 1, **4**. Furthermore, the NMR mediated monitoring of the reaction at 27 °C showed that product **3** was formed rapidly (see Supporting Information). After only 5 min >60% of the precursor **2** had been converted to product **3**, the reaction reaching a plateau at 15 min with around 70% conversion. Increase of reaction temperature served only to accelerate the formation of **4**.

The increased stabilities of TFP esters compared to other active esters have been documented previously for radioiodination reactions.[30] Indeed, direct labeling of 2,3,5,6-tetrafluorophenyl pentafluorobenzoate with TBA-[^{18}F] in DMSO has been reported by Herman et al.[31] In this reaction, a [^{18}F]fluoride for fluoride exchange in the pentafluorophenyl system resulted in a decay corrected yield of 32%. However, this method results in an ^{18}F-product with inherently low specific activity due to isotopic dilution. Importantly, the tetrafluorophenoxy moiety proved not to be sufficiently activated to allow for isotopic exchange with [^{18}F]fluoride as demonstrated in the same work by Herman et al.

After the successful synthesis and isolation of **3**, its utility as an acylating agent was investigated. Two RGD peptides, one with a free amine **5** (NC100717)[32] and the second functionalized with an aminooxy moiety **6**, were chosen as model compounds. The aminooxy functionality was selected in order to investigate whether **3** was capable of reaction with

Table 2. Summary of Tested Conditions for the Radiosynthesis of [^{18}F]**3**[a]

precursor amount (mg)	time (min)	base/amount (mg or μL)[b]	temperature (°C)	PTC[c]	solvent	yield (%) (radio-TLC)[d]	recovery from reaction vessel (%)[e]	yield (%) (TLC × recovery)[f]
7	10	$KHCO_3$/1.6	40	K222	MeCN	77.6	52.2	40.5
10	5	$KHCO_3$/2.1	50	K222	MeCN	63.1	45.7	28.8
7	10	$KHCO_3$/3.0	45	K222	MeCN	19	ND	ND
8.2	10	K_2CO_3/5.0	40	K222	THF	39.2	57.2	22.4
10	10	$KHCO_3$/3.0	40	K222	THF	trace	ND	ND
7	10	$KHCO_3$/1.5	40	K222	MeCN	59	65	38.4
7.3	10	$KHCO_3$/3.0	40	K222	MeCN	17.2	76.9	13.2
7.6	10	$KHCO_3$/3.0	40	K222	MeCN/ *t*-BuOH (2:8)	30.7	86.3	26.5
8	10	TBA-HCO_3/30	40	TBA	DMSO	5.1	70	3.6
10	10	TBA-HCO_3/20	40	TBA	MeCN	65.4	69.5	45.5
9	10	TBA-HCO_3/25	40	TBA	MeCN	55.0	76.8	42.2
15	10	TBA-HCO_3/30	40	TBA	MeCN	67.4	73.7	49.7
9 (n = 4)	10	TBA-HCO_3/30	40	TBA	MeCN/ *t*-BuOH (2:8)	66.3 (5.1)[g]	75.9	50.3 (3.9)[g]

[a] ND = not determined [b] Indicates base used in reaction; K_2CO_3/$KHCO_3$ expressed in mg or μL of a 0.8 M aqueous TBA-HCO_3 solution. [c] Phase-transfer catalyst (PTC) used. [d] Radiochemical yield by radio-TLC. [e] Indicates total amount of radioactivity recovered from reactor decay corrected to start of synthesis. [f] TLC yield multiplied by total recovered radioactivity. [g] Standard deviation.

the aminooxy group at low pH, expanding the scope of the process to encompass site-specific labeling of molecules possessing amino groups. In addition, an $\alpha_v\beta_3$ receptor antagonist **7** belonging to the quinoline-4-one class of peptidomimetics was also synthesized according to Harris et al.[33] Compound **7** was further functionalized with a polyethylene glycol (PEG_{10}) unit, affording **8**. The pharmacophore components of the selected peptides and peptidomimetics have previously been shown to possess affinity for the integrin $\alpha_v\beta_3$ in the low nanomolar range.[32,33] Conjugation with 1.3 equiv of **3** with respect to precursors **5**, **7**, and **8** in a mixture of 0.6 mL of DMSO/acetonitrile/phosphate buffer pH 9 (1:1:1) was used as the standard reaction conditions.[34] As indicated by LC−MS, the conjugates **9**, **10**, and **11** formed rapidly and nearly quantitatively under these conditions with little hydrolysis of the active ester at room temperature after 30 min. The conjugates were purified by reversed-phase preparative HPLC, and the desired products were isolated from the pure fractions by lyophilization. The reaction of **3** with the aminooxy modified peptide **6** at pH 4.3 in a citrate/phosphate/DMSO system proceeded slowly. Improved reaction was observed at elevated temperature and extended reaction time (60 °C;1 h). The conjugate **12** was indeed formed, although yields were significantly lower compared to the aminolysis reactions with **3**. Conjugate **12** was purified and isolated in 16% yield with respect to starting peptide. This strategy may be an alternative for the introduction of active esters site-selectively at low pH in many biomolecules possessing a multitude of free amino functionalities. The structures of the peptides and peptidomimetics with their corresponding conjugates formed with **3** are shown in Figure 1.

Radiochemistry. Azeotropic drying for the preparation of the reactive ^{18}F-complex is described in Materials and Methods.

The radiosynthesis of [^{18}F]**3** from precursor **2** (9 mg, 19 μmol) was first attempted using K222/K_2CO_3 in THF for 10 min at 40 °C. These conditions resulted in formation of precipitates in the reaction mixture and a maximum yield of 39% [^{18}F]**3** from resolubilized [^{18}F]fluoride as evidenced by radio-HPLC and TLC. The less basic bicarbonate salt and acetonitrile as solvent increased the radiochemical yield to 77% (radio-TLC) at 40 °C for 10 min with 52% of the activity recovered from the reaction vessel. Best conditions were found to be those using TBA-HCO_3 as phase-transfer catalyst in acetonitrile/*t*-BuOH (2:8) as reaction solvent. This protocol gave 66.3% incorporation of [^{18}F]fluoride after 10 min at 40 °C with an average of 76% of the total activity recovered from the reaction vessel (n = 4). A summary of all the conditions tested is given in Table 2. Interestingly the *t*-BuOH system gave less precipitation in the reaction mixture and in general cleaner chromatograms. To our knowledge this is the first reported use of a tertiary alcohol used in nucleophilic aromatic substitution reactions with no-carrier-added [^{18}F]fluoride, originally reported by Kim et al. for S_N2-type reactions.[35] The nonradioactive profile observed in the radioactive experiments proved to be very similar to the initial findings with the "cold" fluorination experiments: small amounts of intact precursor (t_R = 3.06 min) with the major impurities being the hydrolyzed product of precursor **2** eluting close to the void, 2,3,5,6-tetrafluorophenol (t_R = 4.31 min), and impurity **4** (t_R = 6.21 min). A typical chromatogram of the crude reaction mixture for the synthesis of [^{18}F]F-Py-TFP is shown in Figure 2.

After establishment of the optimal conditions for the radiosynthesis of [^{18}F]**3**, the next goal was to develop a simple method for purification. By dilution of the crude reaction mixture with water, [^{18}F]F-Py-TFP could be trapped efficiently on an Oasis MCX Plus cartridge with mix-mode reversed-phase and cation-exchange properties. The column was rinsed with 5 mL of water followed by elution of [^{18}F]**3** with a water/acetonitrile mixture (3.5:6.5, 2.1 mL) in >95% radiochemical purity and 70−80% recovery from SPE cartridge. Positively charged species and the highly hydrophobic impurity **4** with the intact active ester were nearly completely retained on the Sep-Pak cartridge, a prerequisite for efficient labeling in the next step. The specific activity for the Sep-Pak purified [^{18}F]F-Py-TFP was calculated from a calibration curve of pure **3** and was calculated to be 4 GBq/μmol. However, these calculations were done from an experiment starting with 125 MBq of [^{18}F]fluoride; hence, higher specific activity of [^{18}F]F-Py-TFP is expected with higher amounts of [^{18}F]fluoride.

Initially, conjugation to peptide precursor **5** in phosphate buffer/DMSO/acetonitrile (pH 9) was studied by addition of aliquots of purified [^{18}F]F-Py-TFP (100 μL, 2−3 MBq). The reaction was studied at various peptide concentrations

Table 3. Radiolabeling of [^{18}F]F-Py-TFP with Peptide Precursor **5** As Analyzed by Radio-HPLC (n = 1 per Time Point)

	radiolabeling yield (%)		
time (min)	0.5 mg/mL peptide	1 mg/mL peptide	3 mg/mL peptide
10	62	84	>95
20	92	>95	>95
30	>95	>95	>95

ranging from 0.5 to 3.0 mg/mL peptide at 40 °C. The conversion of [^{18}F]**3** to [^{18}F]**9** was monitored by radio-HPLC, and the yields were calculated from the percentage of radioactivity peak area of the desired product [^{18}F]**9** relative to starting product [^{18}F]F-Py-TFP.

After 10 min >95% of the labeling reagent [^{18}F]F-Py-TFP had been converted into [^{18}F]**9** at 3 mg/mL of peptide **5**. At peptide concentrations of 1 and 0.5 mg/mL conversion was incomplete after 10 min, reaction times of 20 and 30 min, respectively, were required to achieve conjugation yields of >95%. This clearly demonstrates the dependency of peptide concentration on radiochemical yield.[36,37] The results are summarized in Table 3. The one step protocol and short synthesis time (10 min) of [^{18}F]F-Py-TFP in good yields (50% decay corrected) compare favorably to the normal three step synthesis of [^{18}F]SFB requiring 35−100 min with yields around 25−60% and serve to demonstrate the promise of [^{18}F]F-Py-TFP as a new ^{18}F-prosthetic group for the mild and efficient conjugation of amino groups.[12,18] Moreover, by omission of the time-consuming HPLC purification step customarily employed for [^{18}F]SFB, [^{18}F]F-Py-TFP allows conjugation to biomolecules bearing a free amine under mild conditions (phosphate buffer/organic solvent, pH 9, 40 °C) within 10−30 min in excellent yields (>90%).

Finally, to assess the efficiency of [^{18}F]F-Py-TFP as a labeling agent in a more realistic setting, the process for radiosynthesis of [^{18}F]**9** was performed semiautomatically where only the transfer of reaction mixture to the HPLC injection loop after peptide labeling was handled manually. The synthesis and purification of [^{18}F]F-Py-TFP were performed under the optimal conditions described above. After elution of [^{18}F]F-Py-TFP from the Sep-Pak cartridge back to the reaction vessel, 2 mg of peptide **5** in 1 mL of phosphate buffer/DMSO (1:1), pH 9, was added from a prefilled vial, giving a total volume of 3.1 mL and a final peptide concentration of 0.65 mg/mL (0.5 mM). The reaction mixture was heated to 40 °C for 15 min followed by dilution with water (1.0 mL) and purification by semipreparative reversed-phase HPLC. The fraction containing the desired radioactive product was measured in a dose calibrator and analyzed by radio-HPLC. With this protocol [^{18}F]**9** could be isolated in up to 30% yield based on [^{18}F]fluoride and excellent radiochemical purity (>99%). In a typical set of experiments the decay corrected isolated yields calculated from [^{18}F]fluoride averaged around 22 ± 6% (n = 4). The overall synthesis time including purification was 85 ± 2 min and is likely to be amendable to even shorter synthesis time in a fully optimized automated process. The radiochemical yields compare favorably with other protocols for synthesis of ^{18}F-peptides.[7,38−40] The promising overall radiochemical yield and simplification to the process could therefore represent an alternative methodology for the fully automated production of ^{18}F-peptides. The results of the experiments are summarized in Table 4.

In Vitro Affinity and Stability. A competitive binding assay was used to measure the binding affinity to integrin $\alpha_v\beta_3$ of the four nonradioactive conjugates.[33] Compounds **9**, **10**, **11**, and **12** were found to compete with ^{125}I-echistatin with calculated K_i values in the low nanomolar range (as shown in Table 5), indicating that the 2-fluoronicotinate moiety did not interfere with the binding properties of the pharmacophores.

Table 4. Isolated Radiochemical Yields of [^{18}F]**9** (n = 4)

peptide conjugate	reaction temp (°C)	conjugation time (min)	isolated yield[a] (%)	total synthesis time[b] (min)	RCP[c] (%)
[^{18}F]**9**	40	15	22.5 ± 6.0	85 ± 2	>99

[a] Yield of isolated [^{18}F]fluoropeptide, based on starting [^{18}F]fluoride (corrected for decay). [b] Average time of synthesis measured from incoming [^{18}F]fluoride to end of purification. [c] Radiochemical purity analyzed by radio-HPLC.

Table 5. In Vitro Assay Results for Conjugates **9**, **10**, **11** and **12** in the $\alpha_v\beta_3$ Ea-Hy 926 Assay

conjugate	$\alpha_v\beta_3$ in vitro assay K_i (nM)[a]
9	2.9 ± 0.4
10	19.1 ± 12.2[b]
11	14.8 ± 0.3
12	2.0 ± 0.9

[a] n > 3 experiments. [b] Poor solubility of conjugate **10** in the test medium.

The nonradioactive conjugate **9** was investigated in human plasma to acquire preliminary stability data. The peptide conjugate was incubated in phosphate buffered saline (PBS) and in freshly collected human plasma at 37 °C for 60 min followed by analysis by LC−MS. In both test vehicles no degradation of conjugate **9** was observed. These results are in good agreement with the published in vivo data conducted with other 2-[^{18}F]fluoropyridines systems with particular relevance to defluorination.[26,27]

Conclusion

The starting tetrafluorophenol ester precursor **2** was synthesized in three steps from readily available reagents with no chromatographic purification steps required. An optimized method for the fluorination of the active ester was developed giving good yields of [^{18}F]F-Py-TFP in a single step followed by a convenient purification protocol using an SPE cartridge. The simplification of the radiosynthesis and purification of this prosthetic group was amenable to automation on a commercially available synthesis platform. Conjugation of [^{18}F]F-Py-TFP to an RGD-peptide was found to proceed in good yields and in excellent radiochemical purity.

This new methodology is considered useful for labeling of biomolecules where [^{18}F]SFB is currently employed offering the advantage of a much simpler and rapid radiosynthesis process. In addition, in comparison to the more commonly used ^{18}F-homoaromatics the 2-[^{18}F]fluoropyridines have been shown to produce conjugates with lower log P values potentially resulting in more favorable biodistribution characteristics of the labeled conjugates.[41]

Materials and Methods

Chemicals and solvents of reagent grade obtained commercially were of a purity of ≥95% and were used without further purification. Compounds synthesized were analyzed by HPLC confirming ≥95% purity, unless otherwise stated. All anhydrous chemicals were purchased from Sigma-Aldrich (Norway). HPLC solvents were obtained from Merck KGaA (VWR). Solvents for

all analytic and preparative HPLC were water/0.1% TFA (solvent A) and acetonitrile/0.1% TFA (solvent B). For complete analytical and preparative conditions see Supporting Information.

NMR spectra were run on a Varian Unity Inova 500 spectrometer equipped with a 5 mm ^{1}H-broadband PFG indirect detection probe and on a Bruker Avance III 400 spectrometer equipped with a 5 mm BBFO PFG probe. Mass spectra and analytical HPLC were recorded on a LCQ DECA XP MAX instrument using electrospray ionization (ESI) operated in positive mode at 4.5 kV and scan rate 5500 Da/s coupled to a Finnigan Surveyor PDA chromatography system. High resolution MS of compounds **1**, **2**, and **3** were recorded on a Agilent 1110 series HPLC system coupled to a Agilent MS-TOF (1969A) with a Zorbax SB C18 (50 mm × 4.6 mm, 1.8 μm) using a gradient of 20−90% acetonitrile in 10 mM ammonium acetate over 7.2 min with a flow rate of 1.5 mL/min. Thin layer chromatography (TLC) was run on precoated plates of silica gel $60F_{254}$ (Merck) developed in hexane/ethyl acetate (1:1). Preparative HPLC purifications of nonradioactive reference standards were performed on a Shimadzu system with a Phenomenex Luna C18(2) column (250 mm × 21.2 mm, 5 μm) at a flow rate of 10 mL/min over 60 min with detection at 214 or 254 nm (see Supporting Information). Accurate mass measurements for conjugates **9**, **10**, **11**, and **12** were carried out on a QToF-micro orthogonal acceleration time-of-flight mass spectrometer (Micromass UK Ltd., Manchester, U.K.) coupled with an Agilent 1100 chromatography system (Agilent Technology, Stockport, U.K.) operating in the positive ion ESI mode. Prior to analysis, the instrument was calibrated over a mass range of m/z 100−2000 using sodium iodide. An acquisition rate of 1 spectrum per second was used for all data analyses. The reference compound was iodixanol (3,3′,5,5′-tetrakis(2,3-dihydroxypropylcarbamoyl)-2,2′,4,4′,6,6′-hexaiodo-*N*,*N*′-(2-hydroxypropane-1,3-diyl)-diacetanilide) and was provided by GE Healthcare (Oslo, Norway). Data acquisition and processing were performed using the Masslynx 4.0.

Radiochemistry. Radiochemical syntheses of [^{18}F]**3** and [^{18}F]**9** were performed using the TRACERlab FX F-N (GE Medical Systems) with manual interventions when required. Isocratic reversed-phased preparative HPLC was run on a Phenomenex Luna C18(2) (150 mm × 10 mm, 5 μm), flow 5 mL/min, using 21% solvent B for 20 min with UV detection at 254 nm using the in-built system of the TRACERlab FX F-N. Analytical HPLC was performed on an Agilent system (1100 series) with UV detection in series with a γ-detector (Bioscan flow-count) (see Supporting Information). Instant Imager (Packard BioScience) was used to measure the Radio-TLC scan. [^{18}F]Fluoride was produced by a cyclotron (GE PETtrace 6) using ^{18}O(p,n)^{18}F nuclear reaction with a 16.5 MeV proton irradiation of an enriched [^{18}O]H_2O target.

Synthesis of 6-Chloronicotinic Acid 2,3,5,6-Tetrafluorophenyl Ester (1). A solution of 6-chloronicotinic acid (2.0 g, 13 mmol), tetrafluorophenol (TFP) (2.2 g, 13 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (DCC) (2.60 g, 12.5 mmol) in dioxane (70 mL) was stirred overnight. Dicyclohexylurea (DCU) was filtered off and the solvent removed in vacuo. The residue was dissolved in a minimal volume of hot hexane and immediately filtered. After a short period of time white crystals started to form. To complete the crystallization, the filtrate was stored at 4 °C overnight. The crystals were filtered off and washed twice with ice-cold hexane (2 × 50 mL), affording 6-chloronicotinic acid 2,3,5,6-tetrafluorophenyl ester (**1**) as a white fluffy solid (3.0 g, 79%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.19 (dd, J_1 = 0.6 Hz, J_2 = 2.4 Hz, 1H), δ 8.41 (dd, J_1 = 2.4 Hz, J_2 = 8.3 Hz, 1H), δ 8.07 (dd, J_1 = 0.8 Hz, J_2 = 8.7 Hz, 1H), δ 7.56 (dd, J_1 = 0.7 Hz, J_2 = 8.3 Hz, 1H), δ 7.09 (tt, J_1 = 7.0 Hz, J_2 = 9.8 Hz, 1H). ^{19}F NMR (470 MHz, $CDCl_3$): δ −139.46 (m, 2F), δ −153.65 (m, 2F). Purity (HPLC): 95%, t_R = 3.79 min. HRMS-TOF (m/z): found, 305.9985 $[M + H]^+$, calcd for $C_{12}H_4ClF_4NO_2$ 305.9940.

Synthesis of *N*,*N*,*N*-Trimethyl-5-((2,3,5,6-tetrafluorophenoxy)-carbonyl)pyridin-2-aminium Trifluoromethanesulfonate (2). 6-Chloronicotinic acid 2,3,5,6-tetrafluorophenyl ester (1.0 g, 3.3 mmol) was dissolved in dry THF (15 mL). The solution was filtered into a 35 mL vial and capped with a rubber septum. A steady stream of trimethylamine gas at room temperature was passed through the filtrate under vigorous stirring, allowing excess trimethylamine to escape through a venting needle. After 5 min a white precipitate started to form and the reaction was allowed to proceed for 3 h while maintaining trimethylamine flow. The solid material was filtered off and washed with diethyl ether (100 mL) and cold dichloromethane (50 mL). The solid material (0.53 g, 1.5 mmol) (*N*,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)carbonyl)pyridin-2-aminium chloride) was suspended in dichloromethane (50 mL) under an argon atmosphere by means of ultrasonification. To the vigorously stirred suspension was added trimethylsilyl trifluoromethanesulfonate (0.78 mL, 4.4 mmol) over 5 min. The solution was filtered, and volatile components were removed under reduced pressure. The dry residue was washed with diethyl ether (3 × 50 mL) and dried under vacuum, affording *N*,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)carbonyl)pyridin-2-aminium trifluoromethanesulfonate (**2**) as a white fluffy solid (0.5 g, 32%). ^{1}H NMR (500 MHz, CD_3CN): δ 9.34 (dd, J_1 = 0.8 Hz, J_2 = 2.3 Hz, 1H), δ 8.84 (dd, J_1 = 2.3 Hz, J_2 = 8.7 Hz, 1H), δ 8.07 (dd, J_1 = 0.8 Hz, J_2 = 8.7 Hz, 1H), δ 7.43 (tt, J_1 = 7.3 Hz, J_2 = 10.6 Hz, 1H), δ 3.60 (s, 9H). ^{19}F NMR (470 MHz, CD_3CN): δ −79.72 (s, 3F), δ −140.74 (m, 2F), δ −154.77 (m, 2F). Purity (HPLC): 98%, t_R = 1.76 min. HRMS-TOF (m/z): found, 329.1253 $[M]^+$, calcd for $C_{15}H_{13}F_4N_2O_2$ 329.0913.

Synthesis of 6-Fluoronicotinic Acid 2,3,5,6-Tetrafluorophenyl Ester (3). A mixture of potassium fluoride (7.3 mg, 0.12 mmol) and Kryptofix 222 (59 mg, 0.16 mmol) in dry acetonitrile (1.0 mL) was stirred for 5 min. A solution of *N*,*N*,*N*-trimethyl-5-((2,3,5,6-tetrafluorophenoxy)carbonyl)pyridin-2-aminium trifluoromethanesulfonate (**2**) (50 mg, 0.10 mmol) in dry acetonitrile (0.5 mL was added, and the resulting mixture was stirred for 15 min at room temperature. The reaction mixture was diluted with 2.0 mL water/0.1% TFA, filtered, and purified by reversed-phase preparative chromatography (Phenomenex Luna C18(2) column (250 mm × 21.2 mm, 5 μm), flow rate of 10 mL/min, gradient of 40−80% solvent B over 60 min). The collected fractions were pooled, and acetonitrile was removed under reduced pressure. The aqueous phase was extracted with dichloromethane (3×10 mL). The combined organic phases were washed with water (10 mL), brine (10 mL) and dried (Na_2SO_4). The organic phase was removed in vacuo affording 6-fluoronicotinic acid 2,3,5,6-tetrafluorophenyl ester (**3**) as a waxy off-white solid (10 mg, 37%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 9.10 (dt, J_t = 0.5 Hz, J_d = 2.5 Hz, 1H), δ 8.57 (ddd, J_1 = 2.5 Hz, J_2 = 7.4 Hz, J_3 = 8.6 Hz, 1H), δ 7.13 (ddd, J_1 = 0.5 Hz, J_2 = 3.0 Hz, J_3 = 8.6 Hz, 1H), δ 7.09 (tt, J_1 = 7.1 Hz, J_2 = 9.9 Hz, 1H). ^{19}F NMR (470 MHz, $CDCl_3$): δ −58.31 (dd, J_1 = 2.5 Hz, J_2 = 7.4 Hz 1F), δ −138.75 (m, 2F), δ −152.95 (m, 2F). Purity (HPLC): 99%, t_R = 3.54 min. HRMS-TOF (m/z): found, 290.0249 $[M + H]^+$, calcd for $C_{12}H_4F_5NO_2$ 290.0235.

Preparation of Peptide and Peptidomimetic Precursors (5, 6, 7, and 8). Detailed syntheses of peptide precursors **5** and **6** have been described previously.[33] Quinolin-4-one peptidomimetic **7** was synthesized as described by Harris et al.[32]

Detailed synthesis of PEG_{10} modified peptidomimetic **8** from **7** can be found in Supporting Information.

General Synthesis of ^{19}F-Reference Standards (9, 10, 11, and 12). Complete reaction conditions and analytical data are deposited in Supporting Information.

Synthesis of 6-[^{18}F]Fluoronicotinic Acid 2,3,5,6-Tetrafluorophenyl Ester ([^{18}F]3). Aqueous [^{18}F]fluoride (1 mL, 100−370 MBq) was passed through an anion-exchange resin (Chromafix 30-PS-HCO_3, Machanery-Nagel). The [^{18}F]fluoride was eluted off the resin to the TRACERlab reactor vessel using a mixture of 30 μL of 0.8 M aqueous solution of tetrabutylammonium bicarbonate

(TBA-HCO_3) in water (300 μL) and acetonitrile (300 μL). The solution was concentrated to dryness by heating at 100 °C under reduced pressure and a flow of nitrogen for 2 min. Acetonitrile (0.8 mL) was added twice and evaporated off as above. To the dried TBA-[^{18}F] complex was added **2** (9.0 mg, 19 μmol) dissolved in 1 mL of acetonitrile/*tert*-butanol (2:8). The sealed reaction vessel was heated to 40 °C for 10 min and then analyzed by radio-TLC and HPLC.

Synthesis of Peptide Conjugate [^{18}F]9. The crude reaction mixture containing [^{18}F]**3** was diluted with 2.5 mL of water and loaded onto a preconditioned Oasis MCX Plus Sep-Pak (Waters). The cartridge was rinsed with 5 mL of water, and purified [^{18}F]**3** was eluted back to the reaction vessel using 2.1 mL of water/acetonitrile (3.5:6.5). Peptide **5** (2.0 mg, 1.6 μmol) dissolved in 0.2 M phosphate buffer (pH 9)/DMSO (1:1), 1 mL, was added to the acetonitrile/water solution of purified [^{18}F]**3**, giving a total volume of 3.1 mL. The reaction mixture was heated to 40 °C for 15 min, diluted with water, and purified with preparative HPLC. The fraction containing [^{18}F]**9** was collected, measured in a dose calibrator, and analyzed with radio-HPLC. [^{18}F]**9** coeluted with its authentic reference standard **9**. (HPLC conditions and analytical data are deposited in Supporting Information.)

In Vitro Binding Assay. Membranes from the human endothelial adenocarcinoma cell line EA-Hy926 were prepared, and K_d for ^{125}I-echistatin was determined in the purified membrane fraction. A competitive binding assay was established to measure inhibition constants for each test substance without the need for labeling of the substance itself.[33] ^{125}I-Echistatin was used as the labeled ligand and nonradioactive echistatin as a reference standard. A total of 16 dilutions of test compound (either echistatin or test substance) were prepared and mixed with a combination of the radioactive tracer and membrane prior to incubation for 1 h at 37 °C. Following washing, the bound material was harvested on a filter using a Skatron microharvester. The filter spots were finally excised and counted in a Packard γ-counter. K_i values were calculated from the binding curves using Prism software.

In Vitro Plasma Stability. A solution of **9** (2 mg) in 5 μL of DMSO was added to either 0.4 mL of phosphate buffer saline (PBS), pH 7.4, or freshly collected humane plasma. The peptide was allowed to incubate for 1 h at 37 °C. The two solutions were diluted 1:1 with PBS and ultrafiltered (Ultrafree filter unit 5000 NMWL, Millipore) at 14 000 rpm for 15 min. The resulting filtrate was diluted with water/0.1% TFA and analyzed by LC−MS. Two control runs with blank plasma and PBS were also done as described above.

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Supporting Information Available: HPLC conditions, extended radio-HPLC analytical data, synthesis conditions, affinity measurement, high resolution LC−MS, HPLC spectra, and NMR spectrum of **4**. This material is available free of charge via the Internet at http://pubs.acs.org.

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