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Solid-Phase Synthesis and Fluorine-18 Radiolabeling of CycloRGDyK

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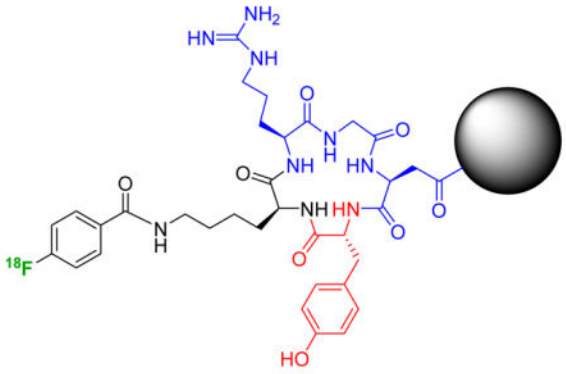
Abstract

Solid-phase peptide synthesis, head-to-tail cyclization, and subsequent radiolabeling provided a reproducible, simple, rapid synthetic method to generate the cyclic peptide radiotracer cRGDyK([^{18}F]FBA). Herein is reported the first on-resin cyclization and ^{18}F-radiolabeling of a cyclic peptide (cRGDyK) in an overall peptide synthesis yield of 88% (cRGDyK(NH_2)) and subsequent radiolabeling yield of 14 ± 2% (decay corrected, n = 4). This approach is generally applicable to the development of an automated process for the synthesis of cyclic radiolabeled peptides for positron emission tomography (PET).

Graphical Abstract



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PET is a minimally invasive imaging technique that has greatly improved medical diagnosis through its ability to detect, diagnose, and follow treatment of cancer and various other diseases by providing three-dimensional (tomographic) whole-body imaging.[1] To visualize the extent of disease *in vivo* with PET, a radiotracer is injected, typically intravenously. Peptide-based radiotracers are becoming a major focus because of the ready ability to be tuned for specificity, affinity, and desirable pharmacokinetic properties, allowing imaging as early as 1 hour post-injection.[2] The biological half-life of peptides also matches the radioactive half-life of ^{18}F (109.77 min).[2] Thus, developing methods to rapidly synthesize radiolabeled peptides is important to facilitate rapid radiotracer development and ultimately translation to the clinic.[1–4]

The arginine-glycine-aspartic acid (RGD)-containing peptides, known to target α_v-integrins have emerged as promising peptide radiotracers for imaging many diseases especially cancer where over expression of α_v-integrins is associated with more aggressive phenotypes.[3–6] Unfortunately, linear RGD-peptides can be prone to proteolytic degradation. Efforts have been made to cyclize them to improve *in vivo* stability and binding selectivity. Kessler and co-workers' pioneered the study of cyclopentapeptide cRGDfV (**1**), which has high affinity and selectivity for the $\alpha_v\beta_3$-integrin.[7] The *N*-methylated valine version of this peptide is now marketed by Merck under the tradename Cilengitide and has shown initial promise as an $\alpha_v\beta_3$-targeted antiangiogenic therapy.[3] Various amino acid replacements and modifications, illustrated in Figure 1, have been studied in attempts to further enhance this cyclopentapeptide's properties, [3,8–11] including an ^{18}F-labeled dimeric derivative of **4**.[12] Several approaches to the radiolabeling of cRGDyK (**4**) have been reported including: the use of activated ester *N*-succinimidyl-4-[^{18}F]-fluorobenzoate ([^{18}F]SFB,[13] activated ester *p*-nitrophenyl-2-fluoropropionate ([^{18}F]NFP,[11] conjugation of 2-[^{18}F]-fluorodeoxyglucose ([^{18}F]FDG),[14] and reaction of aldehydes to form oximes.[15]

The synthesis of cyclic peptides has required head-to-tail cyclizations either done in solution phase or using solid phase peptide synthesis (SPPS).[16–20] These cyclizations often required very dilute conditions, large excess of carboxylic acid activating agents (3–20 equivalents), and long reaction times varying from 6–18 hours up to 5–10 days.[16–20] The reactions, were plagued with side reactions, were poor yielding, lacked the ability to be readily modified for radioisotope incorporation, and in most cases, required multiple purifications. Most synthetic methods towards cyclic peptides suffered from cyclodimerization, oligomerization, or racemization of the C-terminal residue.[16–20] Furthermore, aspartimide formation, *N*-acetylation, piperidine amide formation, and tetramethylguanidinium formation were also observed as byproducts during cyclic peptide synthesis.[16–20]

In order to streamline the synthesis, researchers have focused on the solid phase cyclization step. Cyclopentapeptide, cRGDfK (**2**) was cyclized on solid support using 2 equivalents of 1-[bis(dimethylamino) methylene]-1*H*-1,2,3-triazolo[4,5-b]pyridinium 3-oxid hexafluoro-phosphate (HATU) and a reaction time of 16 hours to afford a 46% yield.[21] Since this synthesis, a fully automated synthesis and cyclization of RGD-peptides has been demonstrated, resulting in 40% yields. However, the automated procedure required a total of 8 equivalents of activating agent and cyclization times between 2–6 hours.[20]

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The use of SPPS in radiochemistry has been beneficial for site-specific addition of a 'prosthetic group' a small synthon to which the radioisotope is attached to in the first step, followed by the coupling with a peptide in a subsequent step.[22–24] Among the wide range of prosthetic groups available,[22,23] [^{18}F]fluoropropionic acid ([^{18}F]FPA) and [^{18}F]fluorbenzoic acid ([^{18}F]FBA), or their respective activated esters [^{18}F]NFP, and [^{18}F]SFB are commonly used to accomplish peptide radiolabeling.[22–27] Recent advances to the introduction of fluorine-18 into peptides include click chemistry, particularly the 1,3-dipolar cycloaddition reaction[28] and its copper-free derivatives[29,30] utilizing ring-strain promoted cycloadditions between cyclooctynes and azides or the use of the inverse-electron demand Diels-Alder reactions between tetrazines and the strained *trans*-cyclooctene.[31,32] Additionally, fluoride capturing reactions based on peptide modifications allow for stable peptide-Si-^{18}F, peptide-B-^{18}F, and peptide-chelator-Al-^{18}F bonds to be generated rapidly and efficiently.[23,33–38] Collectively, these studies indicate a correlation between the prosthetic group and the biodistribution of the radiotracer,[39,40] highlighting the importance of the ability to test different prosthetic groups on a radiolabeled peptide.

Peptide radiolabeling on solid support has been carried out successfully in the Sutcliffe laboratory with the goal to reduce the overall synthetic complexity and number of purifications steps by allowing excess reagents and byproducts to be rinsed away.[25–27] Additionally, SPPS provides a pseudodilution effect which can aid with cyclization, and permit radiolabeling of small amounts of peptide, a current challenge of fluorine-18 radiochemistry. In addition, the solid phase synthetic strategy is amenable to automation. Thus, to further evaluate the capabilities of SPPS-based chemistries for the entire synthetic preparation of peptide radiotracers, this paper describes the use of SPPS-technology to synthesize, cyclize, and radiolabel the clinically relevant cyclic peptide cRGDyK (**4**).

The synthesis of **4** was carried out using fluorenylmethyloxycarbonyl (Fmoc)-chemistry to build the peptide with acid labile side-chain protecting groups on acid sensitive resin (Novasyn®TGA). This resin was selected to (1) avoid the harsh hydrofluoric acid (HF) cleavage as used by the majority of researchers doing on-resin-cyclizations with 4-methylbenzhydryl- amine resin (MBHA) and (2) to help increase cleavage yield while maintaining short cleavage times following the on-resin radiolabeling step.[13–16] The resin was preloaded with the aspartic acid (D), connected via its side-chain and protected as the allyl ester for orthogonal deprotection and cyclization. The use of an orthogonally 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl (ivDde)-sidechain protected lysine provided a handle for attachment of the radiolabel. Protected resin-bound linear yKRGD pentapeptide **6** (Scheme 1) was built by reiterative coupling and deFmocing using 3 equivalents of *N*-terminally Fmoc-protected amino acids, activated with HATU (2.8 equiv) and diisopropylethylamine (DIPEA, 6 equiv) for coupling, and subsequent deprotection with 20% piperidine in *N,N*-dimethylformamide (DMF). After completion of **6**, the allyl ester of aspartic acid (D) was removed by tetrakis (triphenylphosphine) palladium(0) and triphenylsilane, followed by the removal of the *N*-terminal Fmoc. A small-scale test-cleavage, was performed in order to characterize the partially-deprotected linear-peptide of **6** (allyl ester and Fmoc removed); it also, revealed that the ivDde-group migrated to the *N*-terminus during the acidic cleaveage.[41] A reanalysis by HPLC of the purified linear-NH_2-

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yK(ivDde)-RGD exhibited no further ivDde migration, providing further evidence that the cleavage conditions were responsible for the ivDde-migration event.[41] Fortunately, this was only relevant to the test cleavage as the final radiolabeled cyclic peptide would be fully constructed before encountering the acidic cleavage solution.

The on-resin cyclization of partially-deprotected linear-peptide of **6** was done via activation with a single equivalent of HATU and two equivalents of DIPEA to minimize capping and possible dimer/oligomerization byproducts (Scheme 1). The on-resin cyclization was evaluated by removing the peptide from the resin with a mixture of trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and water (95/2.5/2.5) to afford compound **7**, which was then converted to cRGDyK (**4**) by deprotection of lysine's ivDde-group with 2% hydrazine in solution in a nearly quantitative manner. Alternatively, preparation of **4** was also confirmed by first removing the ivDde-group with 2%-hydrazine in DMF, followed by cleavage from the resin and concomitant removal of other side-chain protecting groups using TFA-TIPS-water solution to attain **4** in the same yield (98%) (Scheme 1).

A series of test reactions were performed to monitor the kinetics of the solid-support cyclization and formation of ivDde-protected cRGDyK (**7**) following removal from the resin and HPLC analysis.[41] Contrary to literature data,[16–21] the on-resin-cyclization reaction was very rapid with almost complete transformation of **6** to **7** after 5 min with no observable byproduct formation.[41] Cyclized product (**7**) was obtained in this manner and purified with an overall yield of 90% as determined from the initial loading capacity of the resin. Compound **7** was confirmed by mass spectroscopy and NMR-analysis and conclusively deduced using heteronuclear multiple-bond correlation spectroscopy (HMBC) (Figure 2). The HMBC-NMR spectrum of **7** confirms the point of ring closure for the on-resin cyclization by exhibiting heteronuclear coupling between the α-proton of tyrosine and the carbonyl carbon of aspartic acid. The HMBC-NMR spectrum further shows the 3-bonding coupling through the heteroatom-nitrogen from the ε-protons of lysine's side-chain to the sp^2-hybridzed carbon of ivDde group, indicating that the cyclized product does contain the lysine ivDde protection.

For on-resin radiolabeling and the preparation of the non-radioactive cold fluorine-19 standard, upon cyclization of **6** the ivDde-group of lysine was removed with 2% hydrazine in DMF to obtain the cyclic peptide, with the free ε-amine of lysine (**8**), still attached to the resin (Scheme 2). Compound **8** was first coupled to non-radioactive 4-[^{19}F]-fluoro-benzoic acid ([^{19}F]FBA) to provide a cold standard to confirm the identity of the [^{18}F]-peptide radiolabeled with [^{18}F]FBA. Upon coupling [^{19}F]FBA, global deprotection and simultaneous removal of the peptide from the solid support with TFA-TIPS solution, the non-radioactive product **9** was obtained in 80% yield based on the original loading capacity of the resin (Scheme 2). In addition to being used as a cold standard, cRGDyK([^{19}F]FBA) **9** was also evaluated by a competitive ELISA for binding to integrin $\alpha_v\beta_3$ in the presences of the natural ligand vitronectin.[42] As expected, **9** exhibited good affinity to integrin $\alpha_v\beta_3$ with an IC_{50} = 17 nM. This value matches the one reported for cRGDyK([^{19}F]FBA) **9** in a competition assay against iodine-125 labeled ^{125}I-c(RGDyK) to primary human brain capillary endothelial cells, which was also measured to be 17 nM.[43] Furthermore, for

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cRGDyK (**4**), an IC_{50} of 4 nM is reported in the literature,[44] which is in close agreement to our obtained value of 1 nM for the same compound.

For the synthesis of radiolabeled cRGDyK([^{18}F]FBA) (**10**) (Scheme 3), [^{18}F]FBA was synthesized from ethyl-4-(trimethylammonium triflate) benzoate by reacting [^{18}F]-fluoride in the presence of cryptand, 4,7,13,16,21,24-hexaoxa-1,10-diazabicyclo[8.8.8]-hexacosane (Kryptofix™ [K222]) and potassium carbonate at 100 °C in dimethylsulfoxide (DMSO) for 15 minutes (Scheme 3), followed by hydrolysis of the ethyl ester with sodium hydroxide solution (0.5 M) to give [^{18}F]FBA. The generated [^{18}F]FBA was quenched with hydrochloric acid, cartridge purified (C18-SepPak), concentrated, and added to the 5 mg of pre-swelled peptidyl resin (**8**) (0.49 mg, 0.79 μmol of peptide (**8**)).[27] Following our well established solid-phase radiolabeling procedure for linear peptides,[27] a brief 30 minute coupling of [^{18}F]FBA (96–150 mCi) to **8** via activation of [^{18}F]FBA *in situ* by HATU (5 mg) and DIPEA (10 μL) and a short 30 minute cleavage/deprotection with TFA-TIPS mixture, generated cRGDyK([^{18}F]FBA) (**10**) in 14 ± 2% (n = 4) decay corrected yield. Compound **10** was obtained in >99% radiochemical purity after purification by reverse-phase HPLC in a synthesis time of 90 minutes from [^{18}F]FBA. Notably, even before purification, crude product **10** did not contain radioactive byproducts (Figure 3).

Taken together the overall synthetic approach, including rapid on-resin cyclization and radiolabeling, followed by a single HPLC purification provided the cyclic radiolabeled peptide cRGDyK([^{18}F]FBA) (**10**) in excellent purity and high specific activity of 240±14 GBq/μmol. The radiolabeling results obtained here, compared well to other previously reported radiolabeling reactions of cyclic-RGD peptides. Radiolabeling of **5** with [^{18}F]NFP, proceeded in a 29±5% overall radiochemical yield after decay correction with a 200±18 minutes total reaction time including HPLC purification and required a considerable amount of peptide (5 mgs, 5.7μmol).[11] The solution-phase radiolabeling of cRGDyK(**4**) with [^{18}F]FDG to make a derivative similar to galactoRGD(**5**) was done by modifying the lysine sidechain by attachment of a Boc-aminooxy acetic acid to the ε-amine. Upon removal of the Boc-group, the oxyamine could react with the sugar aldehyde of [^{18}F]FDG.[14] However, this radiolabeling required 1 hour at a low pH = 1.5–2.5 and a temperature of 100 °C to obtain an isomeric mixture of *E*/*Z*-oximes in 41% decay-corrected yield.[14] Similarly, a novel aldehyde, [^{18}F]-fluoro-PEG-nicotinicaldehyde prepared in four steps has been coupled to various cyclic peptide oxyamines in TFA/water solution at 70 °C for 30 min to afford the peptide oximes with radiochemical decay corrected yields ranging from 21–35%.[15] These methods, however, used between 2–5 mg of purified peptide. The most comparable radiolabeling method to the one reported here was carried out by Chen and co-workers where the ε-amine of the lysine in cRGDyK(**4**) (0.1 mg, purified peptide) was labeled with [^{18}F]SFB in less than 2 hours with 20–25% decay corrected yield and specific activity of 230 GBq/μmol.[13]

In conclusion, the presented study has synthesized cRGDyK (**4**), completely on solid support and demonstrated rapid, efficient and convenient on-resin cyclization, in high purity. The presented SPPS and cyclization has circumvented formation of byproducts that have been observed in previous head-to-tail cyclizations: piperidine amide formation, *N*-acetylation, aspartimide formation, cyclodimerizations, and oligiomerizations as well as

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capping due to large excess of uronium derived activating agents.[16–20] In addition the new method improved on previous low yields, shortened the cyclization time, and allows for the precursor peptide to remain fully protected on-resin until after the last synthetic step of radiolabeling, which does not require low pH or harsh conditions and conserves precious and potentially costly peptide material allowing simple purification by a single HPLC injection. More importantly, this procedure is amendable to automation of cyclic-radiolabeled peptides with the ability to readily change the prosthetic group allowing for fine tuning of pharmacokinetic properties. The ability of SPPS to be adapted for continuous-flow systems and automated peptide synthesis allows further exploitation of this technology to the synthesis of radiolabeled peptides with increasing complexity, where rapid site-specific incorporation of the radioisotope is required. To date, this study is to our knowledge the first reported fully on-resin cyclization and radiolabeling of a cyclic-peptide radiotracer. This solid-support cyclization and radiolabeling can be automated for future application of other cyclic-radiolabeled peptides for PET-imaging.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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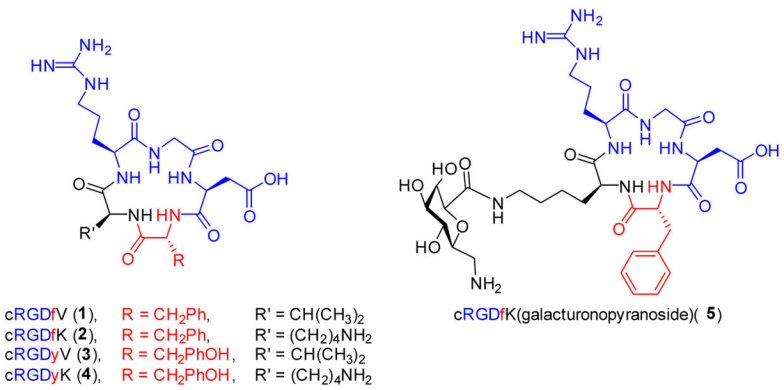

Figure 1.
CycloRGD pentapeptides.

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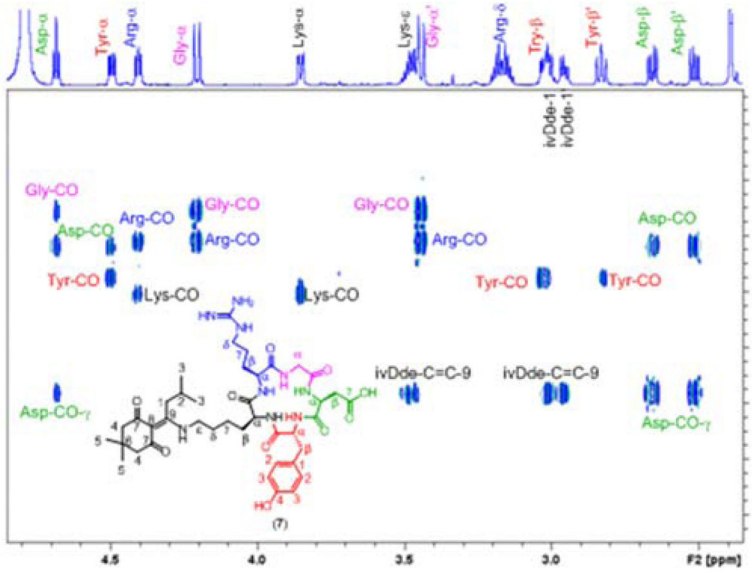

Figure 2.

HMBC-spectrum of compound **7**. The spectrum shows the ivDde sp^2-carbon coupling to ε-protons of lysine and the carbonyl carbon of aspartic acid coupled to the α-proton of tyrosine.

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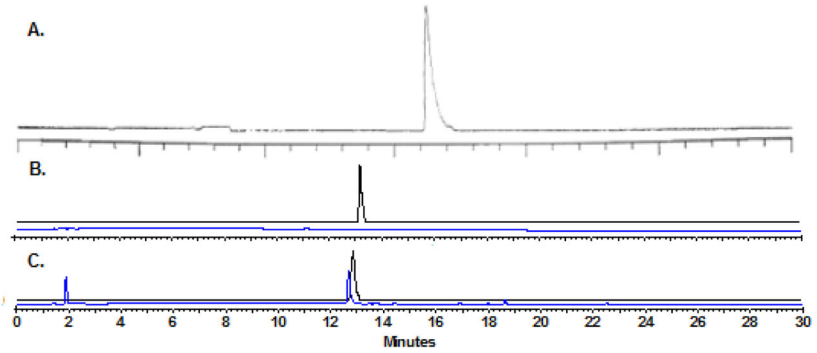

Figure 3.

A. Semi-preparative radioactive HPLC trace of crude **10. B.** Analytical radioactive (black) and UV (220 nm, blue) HPLC trace of purified **10. C.** Analytical radioactive (black) and UV (220 nm, blue) HPLC trace of **10**, co-injected with cold standard **9**.

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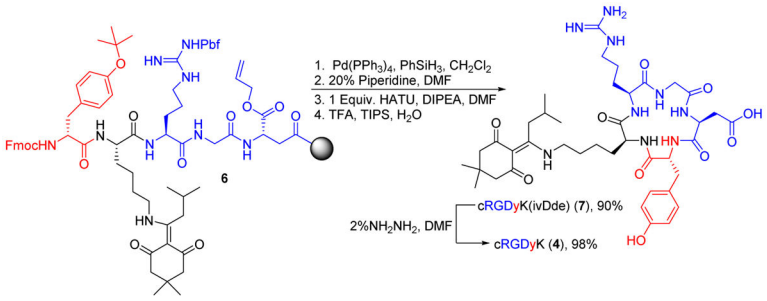

Scheme 1.
Synthesis of cRGDyK(**4**).

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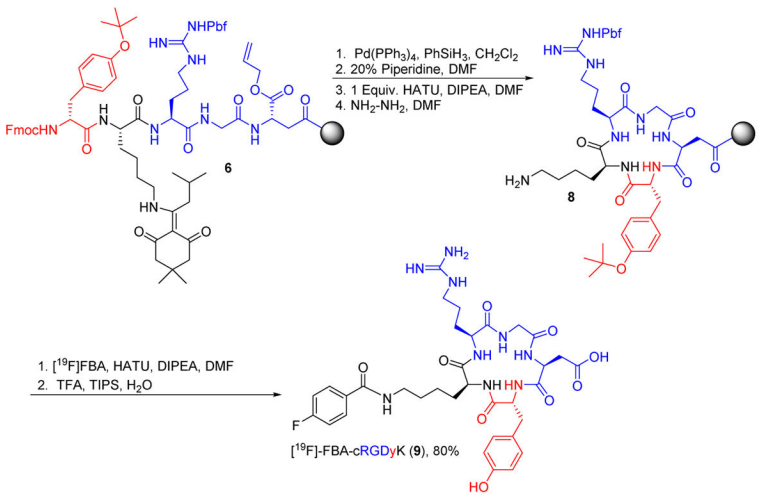

Scheme 2.

Synthesis of cold standard cRGDyK([^{19}F]FBA)(**9**).

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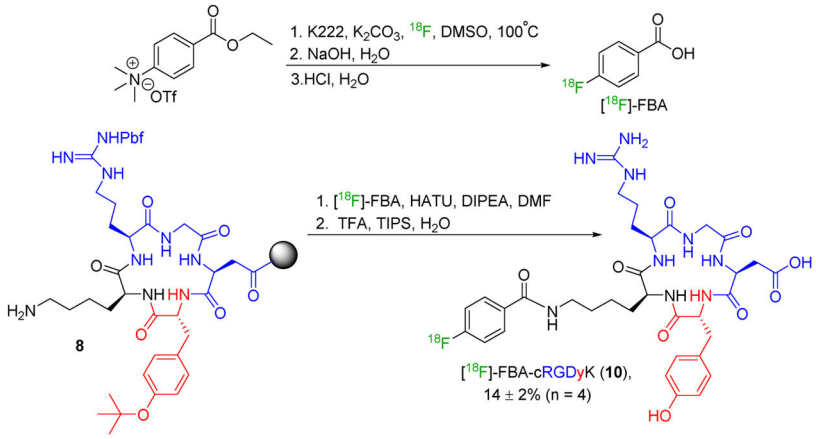

Scheme 3.

Synthesis of [^{18}F]FBA and radiolabeled cRGDyK([^{18}F]FBA)(**10**).