Article

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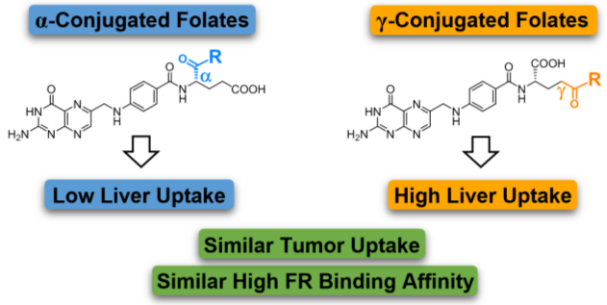
*Bioconjugate Chem.*, **Just Accepted Manuscript** • DOI: 10.1021/acs.bioconjchem.5b00644 • Publication Date (Web): 04 Dec 2015

Table of Contents



# Comparative Studies of Three Pairs of α- and γ-Conjugated Folic Acid Derivatives Labeled with Fluorine-18

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## Abstract

The folate receptor (FR) is upregulated in various epithelial cancer types (FR α-isoform), while healthy tissues show only restricted expression. FR-targeted imaging using folate radiopharmaceuticals is therefore a promising approach for the detection of FR-positive cancer tissue. Almost all folate-based radiopharmaceuticals have been prepared by conjugation at the γ-carboxylic functionality of the glutamate moiety of folic acid. In this work, three pairs of fluorinated α- and γ-conjugated folate derivatives were synthesized and their *in vitro* and *in vivo* properties compared. The syntheses of all the six regioisomers were obtained in good chemical yields using a multi-step synthetic approach including the highly selective Cu(I)-catalyzed 1,3-dipolar cycloaddition. The radiosyntheses of the α- and γ-conjugated $^{18}$F-labeled folate derivatives were accomplished in moderate to good radiochemical yields, high radiochemical purities (> 95%) and specific activities ranging from 25-196 GBq/μmol. *In vitro*, all folate derivatives showed high binding affinity to the FR-α ($IC_{50}$ = 1.4-2.2 nM). *In vivo* PET imaging and biodistribution studies in FR-positive KB tumor-bearing mice demonstrated similar FR-specific tumor uptake for both regioisomers of each pair of compounds. However, FR-unspecific

liver uptake was significantly lower for the α-regioisomers compared to the corresponding γ-regioisomers. In contrast, kidney uptake was up to 50% lower for the γ-regioisomers than for the α-regioisomers. These results show that the site of conjugation in the glutamyl moiety of folic acid has a significant impact on the *in vivo* behavior of $^{18}$F-based radiofolates but not on their *in vitro* FR-binding affinity. These findings may potentially stimulate new directions for the design of novel $^{18}$F-labeled folate-based radiotracers.

## Introduction

The folate receptor (FR) has emerged as an interesting target for imaging FR-positive tumors due to the overexpression of FR-α on a variety of epithelial cancer types including cancer of the ovaries, uterus, lung, breast, kidneys and colon-rectum.[1,2] The expression of FR in normal healthy tissue is limited to a few sites including the kidneys, lung, choroid plexus, salivary glands and the placenta.[1,3,4]

Structurally, folic acid consists of two chemical entities, namely pteroate and glutamate. Together, they form pteroylglutamic acid which is used as a synonym for folic acid (Figure 1).

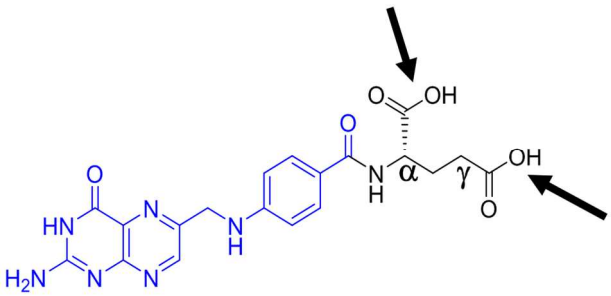



**Figure 1**. Chemical structure of folic acid consisting of pteroic acid (blue) and glutamic acid (black). The two carboxylic functionalities are accessible for derivatization (black arrows).

Chemical modification of folic acid by covalent attachment of chemotherapeutics or prosthetic groups using the pendant approach can in general be accomplished *via* any of the two carboxylic functionalities at the alpha (α) and gamma (γ) positions of the glutamate moiety.[5] However, almost all folate-based chemotherapeutic drugs and radiopharmaceuticals have been prepared *via* derivatization at the γ-position probably because the α-carboxylic acid group in the glutamate part of folic acid is synthetically less easily accessible for conjugation due to steric hindrance.[6,7]

The first biological investigations of α- and γ-regioisomers of folate derivatives were performed by Wang *et al.* who conjugated a deferoxamine chelator to the α- or γ-carboxylic functionalities in the glutamic acid moiety.[8] The authors observed very low or virtually no binding affinity to the FR of the α-regioisomer of deferoxamine folate, whereas the γ-regioisomer exhibited an affinity similar to that of folic acid. One year later, the same group reported on an ethylendiamine-folate derivative, which was labeled with fluorescein isothiocyanate.[9] Similar results with regard to binding affinities were obtained for the α- and γ-conjugated derivatives. The α-regioisomer exhibited virtually no affinity to FR, whereas the γ-regioisomer showed nanomolar affinity similar to that of folic acid.

In contrast to the results reported by Wang *et al.*, Leamon *et al.* observed similar binding affinities to FR-positive cells for proteins conjugated to folic acid either at the α- or γ-carboxylic acid group of the glutamic acid moiety.[10] This observation was also confirmed by Müller *et al.* who showed equal *in vitro* binding affinities to the FR of both α- and γ-regioisomers of $^{99m}$Tc-labeled folate derivatives ($^{99m}$Tc(CO)$_3$-PAMA-folates).[11]

Wedeking *et al.* observed comparable *in vitro* binding affinities of α- and γ-conjugated folate derivatives labeled with $^{153}$Gd or $^{99m}$Tc to FR-positive tumor cells.[5] *In vivo* studies of the α- and γ-folate conjugates in tumor-bearing animals showed equal or greater tumor accumulation of the

α-isomers compared to the corresponding γ-regioisomers. Renal clearance of the α-isomers was significantly higher when compared to the γ-regioisomers. It is, however, not clear whether the results obtained from the radiometallated folates can be generalized to include fluorine-18 labeled folate-based radiopharmaceuticals. In order to gain more insight and a better understanding of the biological behavior of the alpha and gamma regioisomers, we designed and synthesized three pairs of fluorinated α- and γ-conjugated folic acid derivatives. The structures of these six different folate congeners are depicted in Figure 2. Our main goal was to evaluate the *in vitro* and *in vivo* properties of these regioisomers, and to assess whether differences exist in their *in vitro* binding affinities and *in vivo* characteristics.

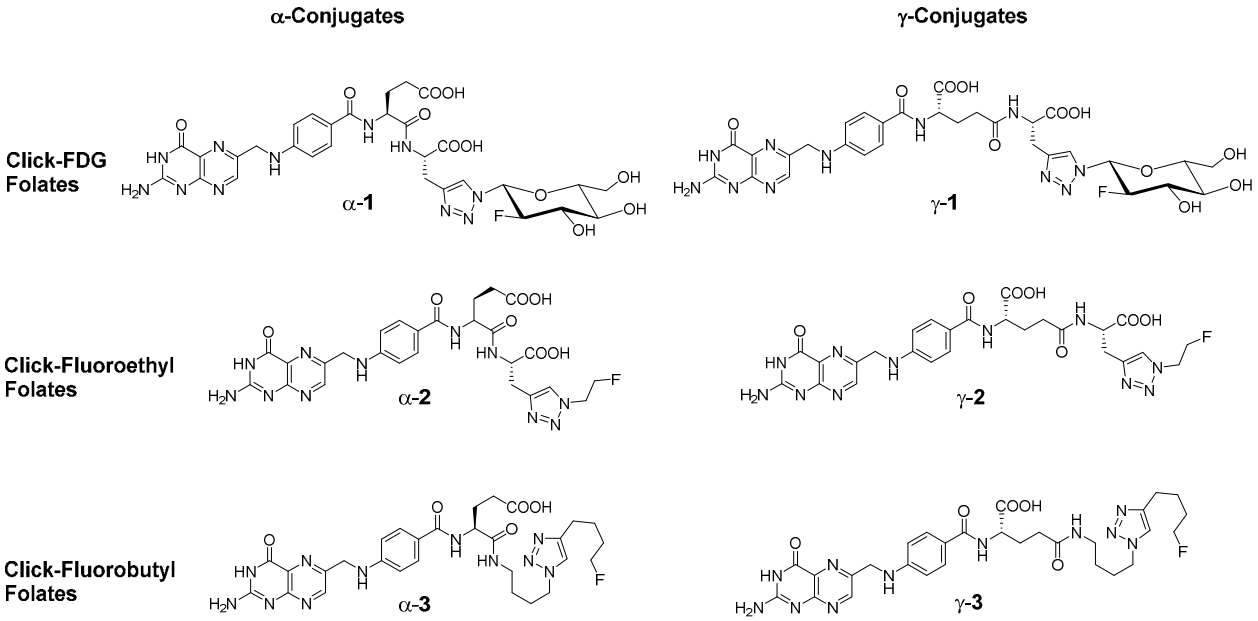



**Figure 2.** Three pairs of fluorinated α- and γ-folate conjugates; click-fluoro-deoxy-glucose folates α/γ-**1** (click-FDG folates), click-fluoroethyl folates α/γ-**2** (click-FE folates) and click-fluorobutyl folates α/γ-**3** (click-FB folates).

## RESULTS

**Synthesis of Non-Radioactive Compounds.** The synthesis of the α-folate alkyne precursor (α-**10**, Scheme 1) was accomplished following the procedure previously described for the γ-isomer (γ-**10**).[12] Coupling of the two commercially available amino acids Boc-Glu(OMe)-OH (**4**) and H-Pra-OMe (**5**) gave glutamate derivative **6** in 80% yield. Acidic Boc-deprotection of intermediate **6** using TFA afforded quantitatively the free amine **7**, which was conjugated in the next step to protected pteroic acid **8** using HBTU as the coupling reagent. Compound **9** was deprotected using NaOH and after preparative HPLC purification of the reaction crude, α-**10** was obtained in 26% yield and purity of > 98%. Compound α-**10** was prepared in four steps in an overall chemical yield of 21%. For the other regioisomer γ-**10**, an overall chemical yield of 31% was obtained as previously reported. The structure of α-**10** was confirmed by NMR spectroscopy and mass spectrometry.

Compounds α-**1** and γ-**1** were synthesized by a Cu(I)-catalyzed cycloaddition between folate alkyne **10** and the azido derivative **11**, which was prepared in 83% chemical yield following a previously reported procedure (Scheme 2).[13] Preparative HPLC purification gave the non-radioactive reference compounds α-**1** and γ-**1** in yields of 62% and 52%, respectively, and chemical purities of both regioisomers were >98%. In Scheme 3 is shown the synthetic route to α- and γ-click-FE folates (α-**2** and γ-**2**). Both regioisomers were obtained from the corresponding folate alkyne building blocks α/γ-**10** and 2-fluoroethylazido compound **12**. 2-Fluoroethyl 4-methylbenzenesulfonate **24** was obtained in 74% yield by treatment of 2-fluoroethanol **23** with tosyl chloride (Scheme S2). Replacing the tosyl leaving group with azide functionality afforded 2-fluoroethyl azide **12** which was directly used for the click-reaction with either precursor α-**10** or γ-**10** to give reference compounds α-**2** and γ-**2**. After semipreparative HPLC purification, the

α- and γ-click-FE folates (α-**2**, γ-**2**) were obtained in 29% and 34% yield, respectively. For radiolabeling, 2-azidoethyl 4-methylbenzenesulfonate **22** (Scheme S1) was synthesized using a one-pot/two-step synthesis in 67% yield after column chromatography.

Non-radioactive α-click-FB folate (α-**3**) was synthesized following the procedure described previously for the γ-isomer (γ-**3**).[14] Treatment of the commercially available *N*-1-Boc-1,4-diamine **13** with *in situ* generated triflyl azide gave the azide intermediate **14** in 96% yield (Scheme 4). Boc-deprotection of intermediate **14** afforded the free amine **15**, which was coupled to protected amino acid BocGlu(OMe)-OH (**4**) to give glutamate derivative **16** in 64% yield. The Boc-protecting group in compound **16** was cleaved using TFA to yield free amine **17** quantitatively, which was coupled to protected pteroic acid **8** using HBTU as a coupling reagent to give compound **18** in 55% chemical yield after semipreparative HPLC purification. Deprotection of **18** using NaOH, followed by semipreparative HPLC purification afforded α-**19** in 63% yield and high purity of >99%. NMR spectroscopy and mass spectrometry confirmed the structure of α-**19**. The synthesis of 6-Fluorohex-1-yne **20** (Scheme S3) was accomplished in a two-step reaction sequence by first reacting hex-5-yne-1-ol **25** with tosyl chloride to give tosylate **26** in 81% yield. Nucleophilic substitution of tosylate **26** with fluoride in a second step delivered alkyne **20** in 25% yield. Compound **20** was directly used for the Cu(I)-catalyzed cycloaddition with folate azides α/γ-**19** in aqueous solution. Non-radioactive reference compounds α-**3** and γ-**3** (Scheme 5) were obtained in 65% and 81% yield, respectively, and a chemical purity of >98% after semipreparative HPLC purification.

**Radiochemistry.** α- and γ-click-[$^{18}$F]FDG folates (α/γ-[$^{18}$F]**1**, Scheme 6) were obtained in a two-step reaction sequence by reacting γ-folate alkynes α-**10** and γ-**10** with

[$^{18}$F]fluoroglucopyranosyl azide ([$^{18}$F]**11**).[12] [$^{18}$F]**11** was synthesized in a two-step reaction sequence (Scheme S4, Supporting Information) and used directly for the Cu(I)-catalyzed cycloaddition to afford α- or γ-[$^{18}$F]**1**. After semipreparative HPLC, both radiolabeled regioisomers α-[$^{18}$F]**1** and γ-[$^{18}$F]**1** were obtained in radiochemical yields of 3-10% and 5-25%, respectively. The radiochemical purity of both α- and γ-[$^{18}$F]**1** was higher ≥ 95% with SA ranging from 30-170 GBq/μmol.[12] Co-injection of the non-radioactive reference compounds α/γ-**1** confirmed the identity of α/γ-[$^{18}$F]**1**.

The radiosyntheses of α- and γ-click-[$^{18}$F]FE folates (α/γ-[$^{18}$F]**2**, Scheme 7) were accomplished in a two step-reaction procedure similar to the radiosyntheses of α/γ-[$^{18}$F]**1**. The first step involved the nucleophilic substitution of the tosylate precursor **21** with [$^{18}$F]fluoride.[15] $^{18}$F-incorporation was greater than 80% after heating at 80 °C for 15 min. Solid phase extraction using a silica cartridge was employed for the removal of non-reacted [$^{18}$F]fluoride and cryptofix/carbonate salts which potentially could interfere with the following click reaction. The second step involving the Cu(I)-catalyzed reaction between radiolabeled azido compound [$^{18}$F]**12** and alkynes α/γ-**10** was carried out at 80 °C for 20 min to afford α- and γ-[$^{18}$F]**2** in 2-4% decay corrected radiochemical yield. At the end of synthesis, 400-800 MBq of the final radioproducts were obtained in up to 2 mL injectable solution, which was sufficient for *in vitro* and *in vivo* experiments. SA ranged from 25–122 GBq/μmol and radiochemical purity was greater 98%.

The radiosyntheses of α- and γ-click-[$^{18}$F]FB folates (α/γ-[$^{18}$F]**3**) were accomplished as shown in Scheme 8. Using hex-5-yn-1-yl 4-methylbenzenesulfonate **26** as the precursor, 6-[$^{18}$F]fluorohex-1-yne [$^{18}$F]**20** was obtained with an $^{18}$F-incorporation yield ranging from 50-70%. In order to remove unreacted [$^{18}$F]fluoride and cryptofix/carbonate salts from the reaction mixture, [$^{18}$F]**20** was distilled at 110 °C into a DMF/DMSO solution containing the folate azide α/γ-**19**. The

subsequent click reaction was carried out in the presence of aqueous solutions of $CuSO_4 \times 5H_2O$ and Na-(+)-*L*-ascorbate at 90 °C for 20 min. After semipreparative HPLC purification and SPE, a physiological product solution of α- or γ-[$^{18}$F]**3** was obtained. Radiochemical purity of α- and γ-[$^{18}$F]**3** was >95% and the SA ranged from 63–196 GBq/μmol. At the end of the synthesis, 2.35–4.17 GBq of radiotracer with a decay corrected yield of 19-23% was obtained.

***In Vitro* Characterization.** To analyze the influence of α- and γ-conjugation on FR-binding affinity, *in vitro* affinity studies were performed with all the three pairs of synthesized reference compounds (α/γ-**1**, α/γ-**2**, α/γ-**3**) using KB cells in a displacement experiment with $^3$H-folic acid. The binding affinities of the compounds were in the same range, independent of the site of conjugation. The obtained affinities were similar to that of folic acid (Table 1).

**Table 1.** Comparison of *in vitro* affinity data of α- and γ- folate regioisomers in comparison to folic acid. ($n$ = 3)

| Compound | $IC_{50}$ [nM] | |
|---|---|---|
| folic acid | 1.1 ± 0.4 | |
| | **α-isomer** | **γ-isomer** |
| click-FDG folates | 1.6 ± 0.2 | 1.5 ± 0.3 |
| click-FE folates | 1.6 ± 0.2 | 1.4 ± 0.2 |
| click-FB folates | 2.2 ± 0.1 | 2.1 ± 0.2 |

The distribution coefficient ($logD_{7.4}$) was determined using the shake-flask method.[16] As expected, both regioisomers of all the three pairs of click-folates, α/γ-[$^{18}$F]**1**, α/γ-[$^{18}$F]**2** and α/γ-[$^{18}$F]**3**, exhibited identical $logD_{7.4}$ values (Table 2).

**Table 2**. Experimentally determined $LogD_{7.4}$ values using the shake-flask method. $LogD_{7.4}$ value of γ-click-FDG folate was taken from the literature.[12]

| Compound | $LogD_{7.4}$ |
|---|---|
| | **α/γ-isomer** |
| click-FDG folates | -4.2 ± 0.1 |
| click-FE folates | -3.0 ± 0.1 |
| click-FB folates | -2.7 ± 0.1 |

**Biodistribution.** The results of the biodistribution studies are summarized in Figure 3, Table S1 and Table S2 (Supporting Information). Biodistribution data for γ-[$^{18}$F]**1** were taken from the literature[12], whereas for γ-[$^{18}$F]**3** new biodistribution experiments were performed. In each experiment, mice were sacrificed at 30, 60 or 90 min p.i.. Mice which received a pre-injection of folic acid for blockage experiments were sacrificed 60 min p.i..

FR-positive tumor uptake of the α- and γ-click-[$^{18}$F]FDG folate (Figure 3, A) after 30 min p.i. was already 9.22 ± 0.30% IA/g and 9.61 ± 1.73% IA/g, respectively. Tumor uptake slightly increased for α-[$^{18}$F]**1** over time to 10.9 ± 0.52% IA/g at 90 min p.i.. In contrast, tumor accumulation of γ-[$^{18}$F]**1** remained at similar values during the time course of the investigation (9.05 ± 2.12% IA/g at 90 min p.i.). Pre-injection of folic acid prior to α-[$^{18}$F]**1** or γ-[$^{18}$F]**1** application efficiently blocked tumor uptake. Liver uptake of α-[$^{18}$F]**1** was 7.84 ± 1.05% IA/g at 30 min, p.i. and decreased to a value of 3.01 ± 0.48% IA/g at 90 min p.i. A significantly higher liver uptake was observed for γ-[$^{18}$F]**1** already at 30 min p.i.(10.8 ± 1.68% IA/g), and remained high over the time course of the study (8.37 ± 1.19% IA/g at 90 min p.i.). High uptake of α-[$^{18}$F]**1** was found in the FR-expressing organs such as kidneys (85.8 ± 8.06% IA/g, 30 min p.i.) and salivary glands (10.1 ± 2.13% IA/g, 30 min p.i.). These values were significantly higher

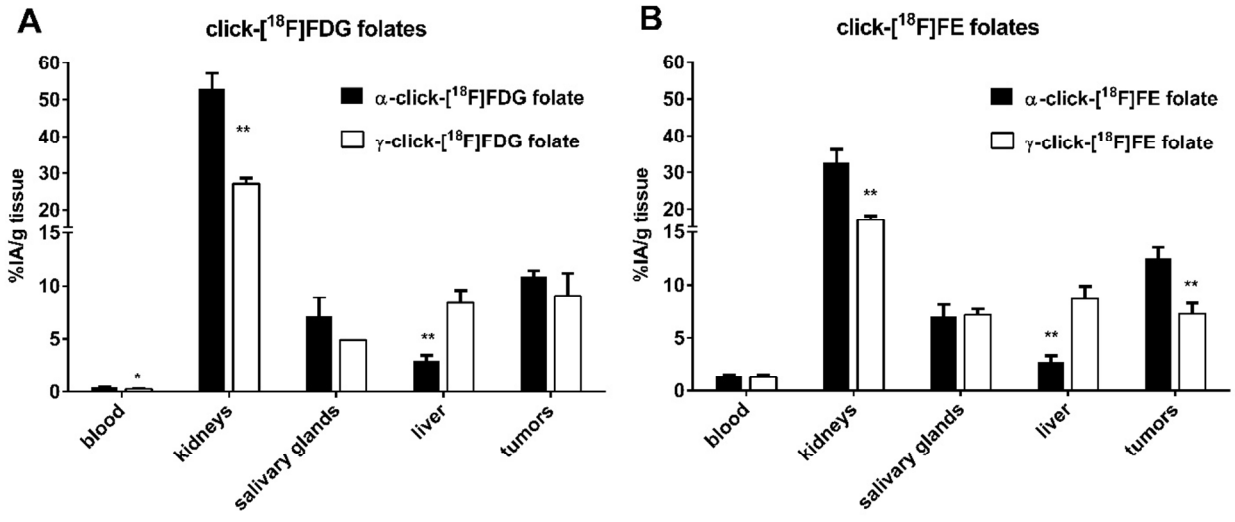
compared to the values obtained for γ-[$^{18}$F]**1** in the kidneys (32.4 ± 1.84% IA/g, 30 min p.i.) and salivary glands (4.61 ± 0.44% IA/g, 30 min p.i.).

A similar biodistribution pattern was observed for the α/γ-click-[$^{18}$F]FE folates (Figure 3, B). Already 30 min after injection of the radiotracer, a significant difference in tumor uptake was found for α-[$^{18}$F]**2** (8.31 ± 0.63% IA/g) and γ-[$^{18}$F]**2** (5.61 ± 1.26% IA/g). Tracer accumulation in the FR-positive tumors increased for both isomers to values of 12.5 ± 1.04% IA/g for α-[$^{18}$F]**2** and 7.24 ± 0.99% IA/g for γ-[$^{18}$F]**2** at 90 min p.i.. Pre-injection of folic acid blocked the tumor uptake of α-[$^{18}$F]**2** and γ-[$^{18}$F]**2** efficiently. Under baseline conditions, liver uptake of α-[$^{18}$F]**2** (7.03 ± 1.02% IA/g) was more than two-fold lower compared to the corresponding γ-regioisomer (16.0 ± 2.69% IA/g) already 30 min p.i.. Radioactivity uptake for both isomers in the liver decreased over time, however, the liver uptake for γ-[$^{18}$F]**2** (8.66 ± 1.19% IA/g) was still significantly higher compared to the corresponding α-isomer (2.77 ± 0.57% IA/g) at 90 min p.i.. As expected, liver uptake of both radiotracers was not affected under blocking conditions, since the FR is not expressed in the liver. Kidney uptake was more than two-fold higher for α-[$^{18}$F]**2** (32.7 ± 3.52% IA/g) compared to γ-[$^{18}$F]**2** (16.9 ± 1.19% IA/g) at 90 min p.i..

Biodistribution data of α/γ-click-[$^{18}$F]FB folates (Figure 3, C) showed low tumor uptake of the α-isomer at all different time points (3.68 ± 0.43% IA/g at 30 min p.i.; 3.31 ± 0.33% IA/g at 60 min p.i.; 3.73 ± 0.62% IA/g at 90 min p.i.). Similar observations were found for the γ-isomer, wherein tumor uptake was also low (2.90 ± 0.63% IA/g at 30 min p.i.; 3.10 ± 1.03% IA/g at 60 min p.i.; 3.22 ± 1.05% IA/g at 90 min p.i.). Under blocking conditions, radioactivity in the tumor xenografts was efficiently reduced. Nonspecific accumulation of α/γ-[$^{18}$F]**3** was observed in the liver, whereby the uptake for the α-[$^{18}$F]**3** (30 min p.i.: 2.44 ± 0.12% IA/g; 60 min p.i.: 1.50 ± 0.34% IA/g; 90 min p.i.: 0.90 ± 0.22% IA/g) was significantly lower compared to γ-[$^{18}$F]**3** (30

min p.i.: 5.12 ± 1.07% IA/g; 60 min p.i.: 3.38 ± 0.63% IA/g; 90 min p.i.: 2.25 ± 0.15% IA/g) at all the investigated three time points. Salivary gland uptake was significantly lower for α-[$^{18}$F]**3** and decreased over time to 2.27 ± 0.76% IA/g at 90 min p.i,, whereas uptake for γ-[$^{18}$F]**3** increased to 9.24 ± 0.73% IA/g at 90 min p.i.. High kidney uptake was found for both regioisomers, whereas the γ-regioismer (32.9 ± 1.67% IA/g) showed a significantly lower uptake compared to the α-regioisomer (44.2 ± 4.03% IA/g;) at 90 min p.i.. High radiotracer accumulation was also found in the gall bladder, urine, and feces, suggesting biliary and renal extraction of α-[$^{18}$F]**3** and γ-[$^{18}$F]**3**.

Tumor-to-liver ratio was significantly higher for all the α-regioisomers when compared to the corresponding γ-regioisomers due to the low liver uptake of the α-conjugated derivatives (Table S2, Supporting Information).



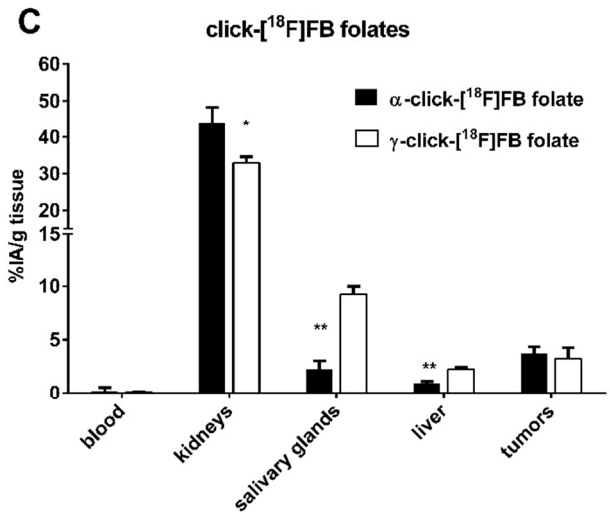
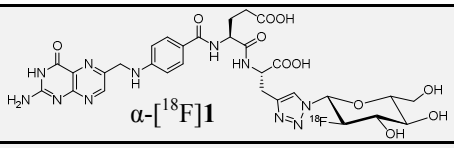
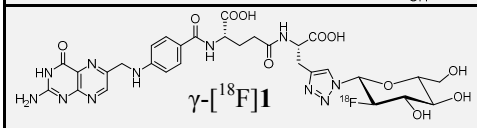
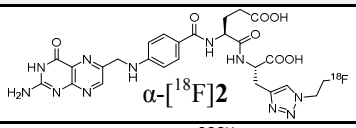
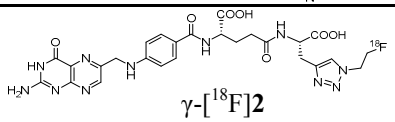
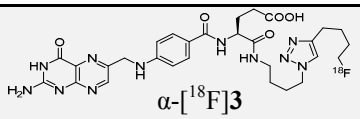
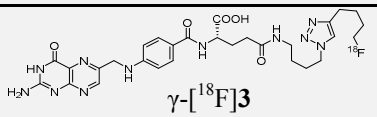



**Figure 3.** Results of biodistribution study of some selected tissues at 90 min p.i. of α/γ-click-[$^{18}$F]FDG folates (A), α/γ-click-[$^{18}$F]FE folates (B) and of α/γ-click-[$^{18}$F]FB folates (C). *$P \leq$ 0.01 and **$P \leq 0.001$.

A summary of some important characteristics of the six regioisomers is presented in Table 3.

**Table 3**. Summary of some important values of the six regioisomers. Biodistribution data are given at 90 min p.i.. The indicated RCY is decay corrected.

| Compound | $LogD_{7.4}$ | $IC_{50}$ value [nM] | RCY [%] | SA [GBq/μmol] | Tumor [% IA/g] | Kidney [% IA/g] | Liver [% IA/g] |
|---|---|---|---|---|---|---|---|
|  α-[$^{18}$F]**1** | -4.2±0.1 | 1.6±0.2 | 3-10 | 30-170 | 10.9±0.52 | 52.9±4.20 | 3.01±0.48 |
|  γ-[$^{18}$F]**1** | -4.2±0.1 | 1.5±0.3 | 5-25 | 52-128 | 9.05±2.12 | 27.1±1.53 | 8.37±1.19 |
|  α-[$^{18}$F]**2** | -3.0±0.1 | 1.6±0.2 | 2-4 | 25-122 | 12.5±1.04 | 32.7±3.52 | 2.77±0.57 |
|  γ-[$^{18}$F]**2** | -3.0±0.1 | 1.4±0.2 | 2-4 | 39-112 | 7.24±0.99 | 16.9±1.19 | 8.66±1.19 |
|  α-[$^{18}$F]**3** | -2.7±0.1 | 2.2±0.1 | 19-23 | 63-71 | 3.73±0.62 | 44.2±4.03 | 0.90±0.22 |
|  γ-[$^{18}$F]**3** | -2.7±0.1 | 2.1±0.2 | 19-21 | 92-196 | 3.22±1.05 | 32.9±1.67 | 2.25±0.15 |

***In Vivo* PET Imaging.** Whole body PET scans were performed with all the $^{18}$F-labeled folates for 30 min starting from 75 min p.i.. In Figure 4 PET/CT images of KB tumor-bearing mice after i.v. injection of the radioligands are shown. High and similar uptake of radiotracer was found in KB xenografts for both α- and γ-click-[$^{18}$F]FDG folates, α-[$^{18}$F]**1** and γ-[$^{18}$F]**1** (Figure 4, panel A). The FR-positive kidneys were also clearly visualized in both cases, whereby the α-regioisomer showed a lower accumulation in the kidneys. Liver uptake was considerably lower for the α-isomer compared to the γ-isomer. For the α/γ-click-[$^{18}$F]FE folates, α-[$^{18}$F]**2** and γ-

[$^{18}$F]**2** (Figure 4, panel B) high tumor uptake was evident. Kidney accumulation was lower for the γ-isomer compared to the α-isomer similar to the results obtained in the biodistribution studies. For α/γ-click-[$^{18}$F]FB folates, α-[$^{18}$F]**3** and γ-[$^{18}$F]**3** (Figure 4, panel C), most of the injected radioactivity was localized in the gastrointestinal tract, the kidneys and the gall bladder, whereas the tumors were not visualized at SUV=14. Similar to regioisomers α-[$^{18}$F]**2** and γ-[$^{18}$F]**2**, lower kidney accumulation was observed for the γ-regioisomer compared to the α-isomer. Pre-injection of folic acid resulted in a remarkably reduced uptake in FR-positive tissues (xenografts, kidneys, salivary glands) for all the six radioligands (data not shown).

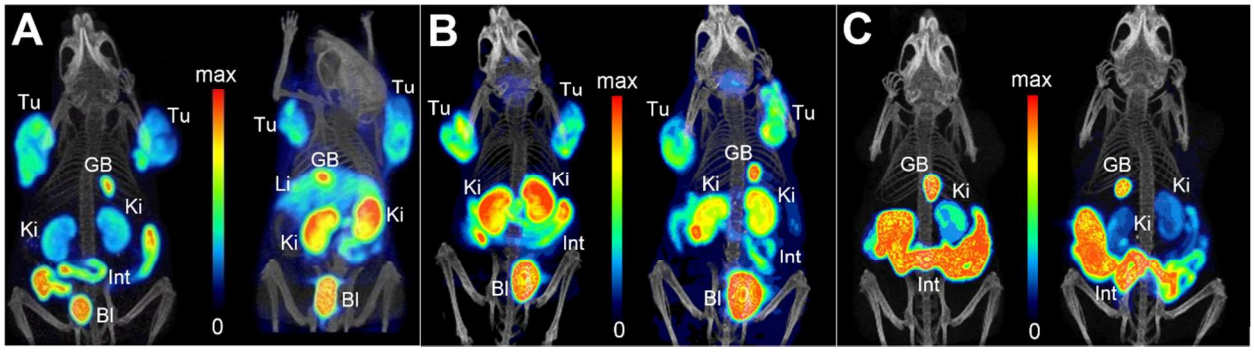



**Figure 4.** Maximum intensity projections of PET/CT scans (averaged from 75–105 min p.i.) performed with KB tumor-bearing mice. A: α-click-[$^{18}$F]FDG folate (left) and γ-click-[$^{18}$F]FDG folate (right), $SUV_{max}$ = 5; B: α-click-[$^{18}$F]FE folate (left) and γ-click-[$^{18}$F]FE folate (right), $SUV_{max}$ = 6; C: α-click-[$^{18}$F]FB folate (left) and γ-click-[$^{18}$F]FB folate (right), $SUV_{max}$ = 14; Tu = KB tumor, Li = liver, Ki = kidney, GB = gall bladder, Bl = urinary bladder, Int = intestines/feces.

**Metabolite Studies.** Metabolite studies were carried out with α-[$^{18}$F]**1**, α-[$^{18}$F]**2** and γ-[$^{18}$F]**2** in mice in order to determine the *in vivo* stability of the tracers. For all the three radioprobes only

intact parent compounds were found 30 min after injection of the tracers (Figures S1, S2 and S3, Supporting Information) similar to results previously reported for γ-[$^{18}$F]**1**.[12]

## DISCUSSION

In this work, we designed and prepared three [$^{18}$F]fluorinated prosthetic groups which were coupled to modified folic acid by chemical linkage to either the α- or γ-glutamyl carboxylate site using click-chemistry to afford three pairs of α- and γ-conjugated folate conjugates with different overall lipophilicities. The synthetic pathways towards α- and γ-folate alkyne precursors, α-**10** and γ-**10**, which were key intermediates in the syntheses of α-**1**, α-**2**, γ-**1** and γ-**2** are shown in Schemes 1, 2 and 3. The overall chemical yields for both alkynes α-**10** and γ-**10** were 21% and 31%, respectively. A similar trend was observed for the α-folate azide α-**19** (21%), which was obtained in a lower chemical yield compared to its corresponding regioisomer, γ-**19** (25%) (Scheme 4). The lower yields of the regioisomers α-**10**, α-**19**, compared to γ-**10** and γ-**19** may be explained by the steric hindrance around the α-position. The highly selective Cu(I)-catalyzed 1,3-dipolar cycloadditions afforded the target compounds α-**1**, γ-**1,** α-**2**, γ-**2,** α-**3**, γ-**3** after HPLC purification in chemical yields ranging from 29-81%. The $IC_{50}$ values of all six compounds were in the range of 1.4 to 2.2 nM, and very similar to that of folic acid (1.1 nM). These results suggest that the site of conjugation in the glutamyl moiety has no dramatic effects on the binding affinities of the resulting α- and γ-folic acid conjugates. These results are in agreement with the recently published crystal structure of FR-α in complex with folic acid which showed that the pteroate moiety in folic acid is buried in the FR, whereas the glutamate functionality is solvent-exposed and sticks out of the pocket entrance allowing it to be conjugated to drugs without adversely affecting binding to the FR-α.[17] These results also confirm

data from Wedeking and coworkers[5], Leamon *et al.*[10], Müller *et al.*[11] and Bettio *et al.*[18] who showed that α- and γ-regioisomers of folic acid conjugates exhibit similar *in vitro* binding affinities to FR.

The radiosyntheses of the $^{18}$F-labeled folate conjugates followed a highly reliable and efficient procedure.[15,19] The first step involved the nucleophilic substitution of a tosylate or triflate leaving group in the prosthetic group with $^{18}$F-fluoride followed by a Cu(I)-catalyzed 1,3-dipolar cycloaddition. α-[$^{18}$F]**1** and γ-[$^{18}$F]**1** were obtained in moderate to good decay corrected radiochemical yields of 3-10% and 5-25%, respectively. High radiochemical yields were also achieved for α-[$^{18}$F]**3** (20-23%, decay corrected) and γ-[$^{18}$F]**3** (19-21%, decay corrected), whereas α-[$^{18}$F]**2** and γ-[$^{18}$F]**2** were obtained in only 2-4% decay corrected radiochemical yields. These low radiochemical yields of α/γ-[$^{18}$F]**2** were not due to the inefficiency of the click reaction but rather due to loss of radioactivity during the semi-preparative HPLC purification of the two tracers. No attempts were made to optimize the semi-preparative HPLC purification since enough radioactivity was obtained for the *in vivo* evaluation of α-[$^{18}$F]**2** and γ-[$^{18}$F]**2.** All the six radiotracers were isolated in high radiochemical purity (> 95%) after HPLC purification and SA ranged from 25-196 GBq/μmol.

In metabolite studies, α-[$^{18}$F]**1**, γ-[$^{18}$F]**1**, α-[$^{18}$F]**2** and γ-[$^{18}$F]**2** exhibited high *in vivo* stability, since only intact parent compounds were found in samples taken 30 min after injection of the radiotracers (Figures S1, S2 and S3, Supporting Information). Previous metabolite studies with γ-[$^{18}$F]**1** and γ-[$^{18}$F]**3** showed no radioactive metabolites or defluorination[12,14] therefore in this study no metabolite studies were performed for these two radioligands.

PET imaging experiments and biodistribution data showed similar tumor uptake values for the respective α- and γ-regiosiomers whereby the α-regioisomers showed slightly higher tumor

uptake values than the corresponding γ-regioisomers. A possible explanation might be the lower hepatobiliary clearance of the α-regioisomers compared to the γ-regiosiomers since a lower accumulation of the α-regioisomers in the liver was observed. Regioisomer α-[$^{18}$F]**2** exhibited the highest tumor uptake value of 12.5 ± 1.04% IA/g at 90 min p.i.. Of all the regioisomers investigated α-[$^{18}$F]**3** and γ-[$^{18}$F]**3** showed the lowest tumor uptake. We attribute this low uptake to the lower hydrophilicity of these regiosiomers ($LogD_{7.4}$ = -2.7 ± 0.1) compared to α/γ-[$^{18}$F]**1** ($LogD_{7.4}$ = -4.2 ± 0.1) and α/γ-[$^{18}$F]**2** ($LogD_{7.4}$ = -3.0 ± 0.1).

An interesting finding of this study is that radioactivity accumulation in a non-targeted tissue such as the liver was generally lower for all the α-regioisomers when compared to their corresponding γ-isomers. As a consequence, the tumor-to-liver ratios were considerably higher for the α-regioisomers than the corresponding γ-regioisomers for all the three pairs of the folate conjugates. Since FR is not expressed in liver cells, we speculate unspecific carrier-mediated uptake *via* organic anion transporters or proton-coupled folate transporter (PCFT) might be operating. The PCFT is mainly expressed in the liver and the intestinal tract.[20,21] We presume that the steric constraints imposed on the α-regioisomers through conjugation to the α-glutamyl moiety render the α-regioisomers less accessible to the PCFT and other unspecific carriers. This assumption is purely a speculation and needs to be verified in future *in vitro* studies. The low uptake of the α-isomers in the liver has an advantage from an imaging point of view because it would allow delineating FR-positive tumors that are located close to the liver. Furthermore, the assessment of liver metastases would be feasible due to a reduced background activity or spill-over from the liver.

The detection of liver metastasis with the clinically used γ-conjugated folate radiopharmaceutical $^{99m}$Tc-EC20 is not possible due to substantial liver uptake in humans, although mice did not

show liver uptake.[22,23] These results suggest that the pharmacokinetics of folate radiopharmaceuticals can differ considerably in mice and humans. However, the use of α-conjugated folate radiopharmaceuticals could be a possible way to overcome the problem of unspecific liver uptake. Clearly, the results of this study have shown that the site of conjugation in folic acid of folate-based radiopharmaceuticals has an impact on the *in vivo* characteristics of the $^{18}$F-labeled folic acid conjugates.

## CONCLUSION

It could be shown for the first time that the site of conjugation in folic acid has an impact on the *in vivo* distribution pattern of $^{18}$F-labeled folate conjugates but not on the *in vitro* binding affinity towards FR. It is expected that these new findings may stimulate new directions for the design of novel fluorinated folate-based PET radiotracers but may also have an impact on the synthesis of folate-based ligands for other applications.

## EXPERIMENTAL PROCEDURES

**General.** Reagents and solvents were purchased from Bachem, Sigma-Aldrich Chemie GmbH, Acros Organics and VWR International AG and used without further purification. The $N^2$-*N*,*N*-dimethylaminomethylene-10-formylpteroic acid (protected pteroic acid, **8**) was generously provided by Merck & Cie (Schaffhausen, Switzerland). Nuclear magnetic resonance spectra were recorded on a Bruker 400 or 500 MHz spectrometer with the corresponding solvent signals as an internal standard. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (0.00 ppm). Values of the coupling constant (*J*) are given in hertz (Hz); the following abbreviations are used in this section for the description of the $^1$H NMR: singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m), doublet of doublets (dd), and bs (broad signal). The chemical shifts of complex multiplets are given as the range of their occurrence. High-resolution mass spectra (HR-MS) were recorded with a Bruker FTMS 4.7 T BioAPEXII (ESI).

Preparative HPLC was performed with a Merck-Hitachi system, equipped with a D7000 interface, L-7400 UV detector and a L-7150 pump, using a reversed-phase column (Ultisil™, former named Ultimate, C18 5 μm, 21.2×150 mm, Welch Materials) at a flow rate of either 15 mL/min (α/γ-**19**) or 18 mL/min (α/γ-**1** and α/γ-**10**).

Analytical and semipreparative HPLC was performed with a Merck-Hitachi system, equipped with a D-7000 interface, L-7400 UV detector and a L-7100 pump, using a reversed-phase column (analytical columns: α/γ-**1**: Gemini C18, 5 μm, 4.6×250 mm, Phenomenex; α/γ-**2**: XBridge C18, 5 μm, 4.6×150 mm, Waters; α/γ-**3**: Sunfire C18, 5 μm, 4.6×150 mm, Waters; semipreparative columns: α/γ-**1**: Gemini C18, 5 μm, 10×250 mm, Phenomenex; α/γ-**2**: XBridge C18, 5 um, 10×150 mm; α/γ-**3**: Sunfire C18, 5 μm, 10×150 mm). Analytical HPLC was

performed at a flow rate of 1 mL/min, whereas the flow rate for the semipreparative HPLC was either 3 mL/min (α/γ-[$^{18}$F]**1**) or 4 mL/min (α/γ-[$^{18}$F]**2** and α/γ-[$^{18}$F]**3**).

Analytical radio-HPLC was performed on an Agilent 1100 series HPLC system, equipped with a 100 µL-loop and a GabiStar radiodetector (Raytest). An analytical column (Gemini C18, 5 µm, 4.6×250 mm, Phenomenex) was used at a flow rate of 1 mL/min.

Purification of the radiolabeled products were performed by using a semipreparative radio-HPLC system equipped with a Smartline Pump 1000, Smartline Manager 5000, Smartline UV detector 2500 (Knauer), 5 mL-loop and a GabiStar radiodetector (Raytest). A semipreparative HPLC column (Gemini, C18, 5 µm, 250×10 mm, Phenomenex) was used at a flow rate of either 3 mL/min (α/γ-[$^{18}$F]**1**) or 4 mL/min (α/γ-[$^{18}$F]**2** and α/γ-[$^{18}$F]**3**). All the semipreparative and analytical HPLC methods are available in the Supporting Information.

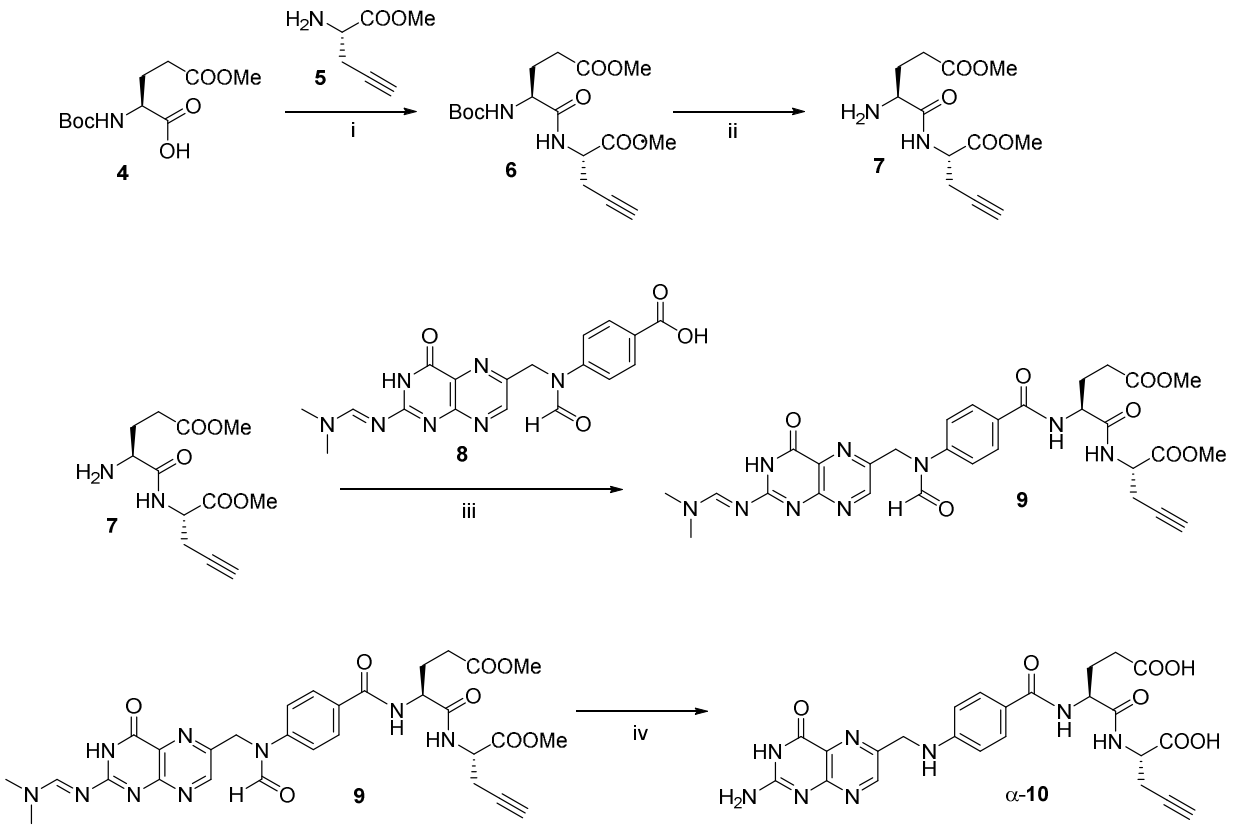
For the radiometabolite study, an ultraperformance liquid chromatography (UPLC, Waters) system with an Acquity UPLC BEH C18 column (2.1×50 mm, 1.7 µm, Waters) and an attached coincidence detector (FlowStar LB513, Berthold) was used with 50 mM $NH_4HCO_3$ solution (solvent A) and acetonitrile (solvent B) as solvents and a gradient from 0-0.5 min 100% A, 0.5-3.5 min 100-30% A, 3.5-3.9 min 30% A at a flow rate of 0.5 mL/min for α-[$^{18}$F]**1**. For the analysis of α/γ-[$^{18}$F]**2**, 10 mM $NH_4HCO_3$ solution (solvent A) and acetonitrile (solvent B) as solvents were used and a gradient from 0-0.3 min 100% A, 0.3-1.0 min 100-85% A, 1.0-1.6 min 85% A, 1.6-2.8 min 85-40% A, 2.8-3.5 min 40% A, 3.5-3.7 min 40-100% A and 3.7-4.0 min 100% A at a flow rate of 0.6 mL/min.

## Organic Syntheses

**Synthesis of α- and γ-Alkyne Folate Isomers.** The synthesis of γ-alkyne folate was performed as previously described.[12] The α-isomer was synthesized following the same synthetic procedure (Scheme 1). Boc-Glu(OMe)-OH×DCHA **4** (487 mg, 1.10 mmol) was dissolved in anhydrous DMF (10 mL) and $NEt_3$ (612 µL, 4 eq.) and HBTU (492 mg, 1.30 mmol) were added at 0 °C. This mixture was added to a solution of H-Pra-OMe×HCl **5** (147 mg, 1.16 mmol) in anhydrous DMF (4 mL) and $NEt_3$ (612 µL, 4 eq.) at 0 °C and stirred for 1 h at 0 °C. The solution was then allowed to warm up to room temperature and stirring was continued overnight. The reaction mixture was diluted with 1 M citric acid (50 mL) and extracted with EtOAc (50 mL). Thereafter the organic phase was rinsed with brine, dried over $Na_2SO_4$, filtered and concentrated under reduced pressure. Purification was achieved by flash chromatography on silica gel with $CH_2Cl_2$/MeOH (50:1), providing the desired product **6** as a clear oil (326 mg, 80%). For removal of the Boc group, compound **6** (270 mg, 0.73 mmol) was dissolved in $CH_2Cl_2$ (4.5 mL) and trifluoroacetic acid (0.5 mL) was added. The mixture was stirred at room temperature for 5 h and was then concentrated under reduced pressure to yield the TFA salt of amine **7** as a yellow oil (198 mg, quantitative). At 0 °C HBTU (314 mg, 0.83 mmol) was added to a suspension of $N^2$-N,N-dimethylaminomethylen-10-formyl-pteroic acid **8** (246 mg, 0.62 mmol) in anhydrous DMF (2 mL) and $NEt_3$ (165 µL, 2 eq.) and the reaction mixture was stirred for 5 min. The resulting orange solution was added at 0 °C to a solution of compound **7** (160 mg, 0.59 mmol) in anhydrous DMF (3 mL), containing $NEt_3$ (165 µL, 2 eq.). Thereafter, the clear yellow solution was stirred at 0 °C for 4 h and then allowed to warm up to room temperature and stirred overnight. Removal of volatile compounds under reduced pressure and purification of the residue by flash chromatography on silica gel with $CH_2Cl_2$/MeOH (10:1) provided compound **9** as a

yellow oil, which was directly deprotected, using 1 M NaOH (1 mL). The solution was stirred over night at room temperature and thereafter extracted with small amounts of EtOAc. The pH of the aqueous phase was adjusted to pH 8 with 2 M HCl. The solution was diluted with 50 mM $NH_4HCO_3$ and α-**10** was purified *via* preparative HPLC using 50 mM $NH_4HCO_3$ solution (solvent A) and MeCN (solvent B) as solvent system and a gradient as follows: 0-20 min: 100-90% A, 20-22 min 90% A, 22-28 min 90-40% A, 28-34 min 90% A. After preparative HPLC, the α-alkyne folate precursor α-**10** was obtained in an overall yield of 16% yield, whereas the yield of γ-**10** was 19%. Both precursor isomers (α-**10** and γ-**10**) were used for the preparation of radioactive labeled α- and γ-click-[$^{18}$F]FDG folate and α- and γ-click-[$^{18}$F]FE folate and their non-radioactive reference compounds.

**Scheme 1.** Synthesis of α-folate alkyne[a]



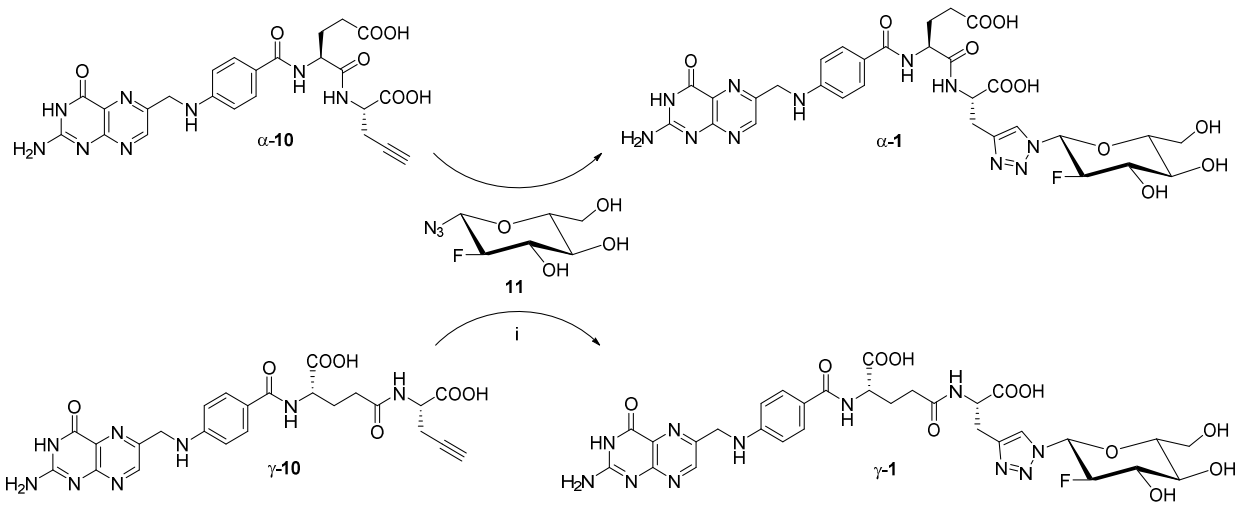
[a](i) HBTU, $Et_3N$, DMF, 0 °C to rt, 20 h, 80%; (ii) TFA/$CH_2Cl_2$ (1:9), rt, 5 h, quant.; (iii) HBTU, $Et_3N$, DMF, 0 °C to rt, 18 h; (iv) aq. NaOH (1 M), rt, 15 h, 21% (over four steps).

**Synthesis of 2-Deoxy-2-fluoroglucopyranosyl azide.** Compound **11** was prepared according to the procedure reported by Maschauer *et al*..[13]

**Synthesis of α- and γ-Click-FDG Folates.** The synthesis of γ-click-FDG folate (γ-**1**) was performed as previously described.[12] Preparation of α-click-FDG folate (α-**1**) followed the same procedure (Scheme 2). 2-deoxy-2-fluoroglucopyranosyl azide **11** (18 mg, 86 μmol) was reacted with α-folate alkyne α-**10** (22 mg, 40 μmol) in t-BuOH/$H_2O$ (1:1, 1.8 mL), a 100 mM aqueous solution of $Cu(OAc)_2 \times H_2O$ (0.1 eq., 40 μL) and a 100 mM aqueous solution of Na-(+)-*L*-ascorbate (0.2 eq., 80 μL). The solution was shaken at 500 rpm at room temperature for 90 min until complete conversion. For isolation of α-**1**, the mixture was injected into a preparative HPLC, applying the same method as for the purification of compound α-**10**.

After preparative HPLC, α-**1** and γ-**1** were obtained in 62% (18 mg) and 52% (7.2 mg) yield, respectively, and purities were determined to be > 95%. α-**1**: $^1$H NMR ($D_2O$/NaOD) δ/ppm 8.63 (s, 1H), 7.98 (s, 1H), 7.67 (d, $^3J$ = 8.4 Hz, 2H), 6.86 (d, $^3J$ = 8.8 Hz, 2H), 5.69 (dd, $^3J_1$ = 8.8 Hz, $^4J_2$ = 2.4 Hz, 1H), 4.66 (t, $^3J$ = 9.2 Hz, 1H), 4.62 (s, 2H), 4.49 (q, $^3J$ = 4.4 Hz, 1H), 4.43 (dd, $^3J_1$ = 5.2 Hz, $^3J_2$ = 4.4 Hz, 1H), 3.94 – 3.83 (m, 1H), 3.71 (dd, $^3J_1$ = 12.4 Hz, $^4J_2$ = 2.2 Hz, 1H), 3.65 – 3.53 (m, 2H), 3.52 – 3.45 (m, 1H), 3.34 (dd, $^2J_1$ = 14.8 Hz, $^3J_2$ = 4.4 Hz, 1H), 3.11 (dd, $^2J_1$ = 14.8 Hz, $^3J_2$ = 9.2 Hz, 1H), 2.30 – 2.24 (m, 2H), 2.12 – 2.02 (m, 1H), 2.02 – 1.90 (m, 1H); HR-MS ($ES^+$) calculated for $C_{30}H_{35}FN_{11}O_{11}$: 744.2496; found: 744.2497.

**Scheme 2.** Synthesis of α- and γ-click-FDG folate reference compounds[a]




[a](i) t-BuOH/$H_2O$, $Cu(OAc)_2 \times H_2O$, Na-(+)-*L*-ascorbate, 90 min, rt; α-**1**: 62%; γ-**1**: 52%

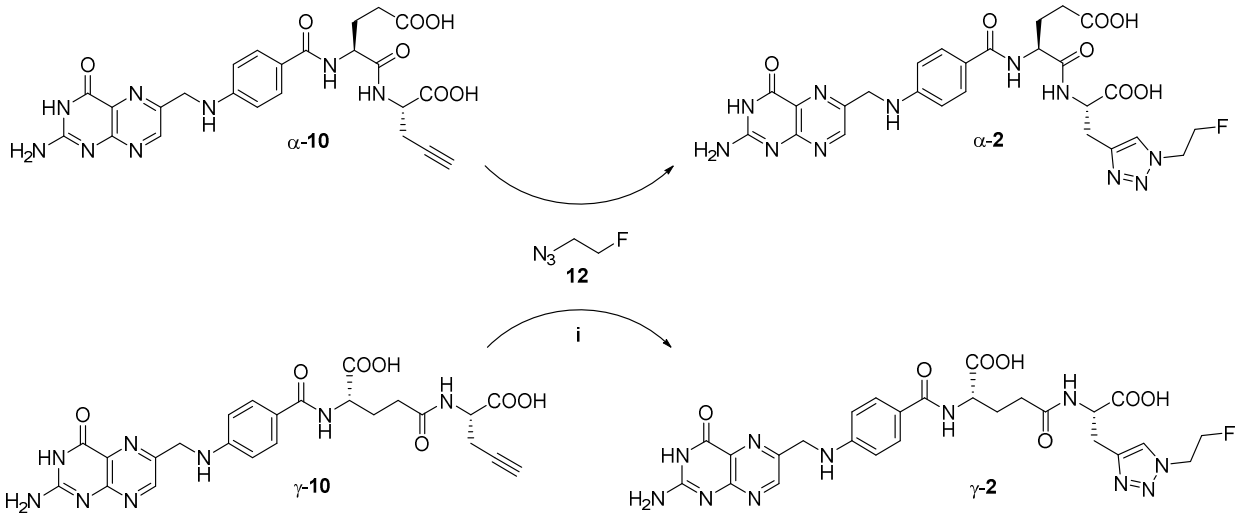
**Synthesis of 2-Azidoethyl 4-methylbenzenesulfonate.** Compound **22** was prepared according to a published procedure (Scheme S1, Supporting Information).[24]

**Synthesis of 2-Fluoroethyl azide.** Compound **12** was prepared in analogy to procedures published in literature (Scheme S2, Supporting Information). [25,26]

**Synthesis of α- and γ-Click-FE Folates.** The syntheses of α- and γ-click-FE folate (α/γ-**2**) are shown in Scheme 3. α- or γ-Folate alkyne α/γ-**10** (5.4 mg, 0.01 mmol) was dissolved in $H_2O$ (400 µL) and a 100 mM aqueous solution of $Cu(OAc)_2 \times H_2O$ (0.1 eq., 10 µL) and a 100 mM aqueous solution of Na-(+)-*L*-ascorbate (0.2 eq., 20 µL) were added, followed by 100 µL of 2-fluoroethyl azide solution (**12**). The reaction mixture was shaken at 900 rpm on an Eppendorf shaker at room temperature. After two hours, additional 50 µL of the 2-fluoroethyl azide solution were added and reacted for additional 60 min. The progress of the reaction was followed by

analytical HPLC using 50 mM $NH_4HCO_3$ solution (solvent A) and MeCN (solvent B) as a solvent system with a gradient from 0-4 min 100% A, 4-25 min 100-80% A, 25-26 min 80-40% A, 26-35 min 40% A, 35-36 min 40-100% A, 36-45 min 100% A. After completion of the reaction, semi-preparative HPLC was performed applying the same gradient and solvent system used for the analytical HPLC to yield α-**2** (1.8 mg, 29%, purity >98%) or γ-**2** (2.1 mg, 34%, purity >99%). α-**2**: $^1$H NMR (DMSO-$d_6$/$D_2O$) δ/ppm 8.64 (s, 1H), 7.82 (s, 1H), 7.65 (d, $^3J$ = 8.8 Hz, 2H), 6.64 (d, $^3J$ = 8.8 Hz, 2H), 4.83 – 4.74 (m, 1H), 4.71 – 4.62 (m, 1H), 4.59 – 4.54 (m, 1H), 4.48 (s, 2H), 4.31 (dd, $^3J_1$ = 8.8, $^3J_2$ = 5.6 Hz, 1H), 4.17 – 4.09 (m, 1H), 3.16 (dd, $^2J_1$ = 14.8, $^3J_2$ = 4.8 Hz, 1H), 2.95 (dd, $^2J_1$ = 14.8, $^3J_2$ = 7.6 Hz, 1H), 2.26 (t, $^3J$ = 7.6 Hz, 2H), 2.01 – 1.90 (m, 1H), 1.89 – 1.78 (m, 1H); HR-MS ($ES^+$) calculated for $C_{26}H_{29}FN_{11}O_7$: 626.2230; found: 626.2231; γ-**2**: $^1$H NMR (DMSO-$d_6$/$D_2O$) δ/ppm 8.64 (s, 1H), 7.77 (s, 1H), 7.61 (d, $^3J$ = 8.8 Hz, 2H), 6.64 (d, $^3J$ = 8.8 Hz, 2H), 4.79 (t, $^3J$ = 4.8 Hz, 1H), 4.65 (dt, $^3J_1$ = 9.2, $^4J_2$ = 4.4 Hz, 2H), 4.56 (t, $^3J$ = 4.4 Hz, 1H), 4.48 (s, 2H), 4.31 (dd, $^3J_1$ = 8.4, $^3J_2$ = 4.8 Hz, 1H), 4.17 (dd, $^3J_1$ = 8.8, $^3J_2$ = 4.4 Hz, 1H), 3.10 (dd, $^2J_1$ = 14.8, $^3J_2$ = 4.8 Hz, 1H), 2.91 (dd, $^2J_1$ = 14.8, $^3J_2$ = 8.4 Hz, 1H), 2.21 – 2.11 (m, 2H), 2.02 – 1.91 (m, 1H), 1.91 – 1.78 (m, 1H); HR-MS ($ES^+$) calculated for $C_{26}H_{29}FN_{11}O_7$: 626.2230; found: 626.2230.

**Scheme 3.** Synthesis of reference compounds α- and γ-click-FE folate[a]



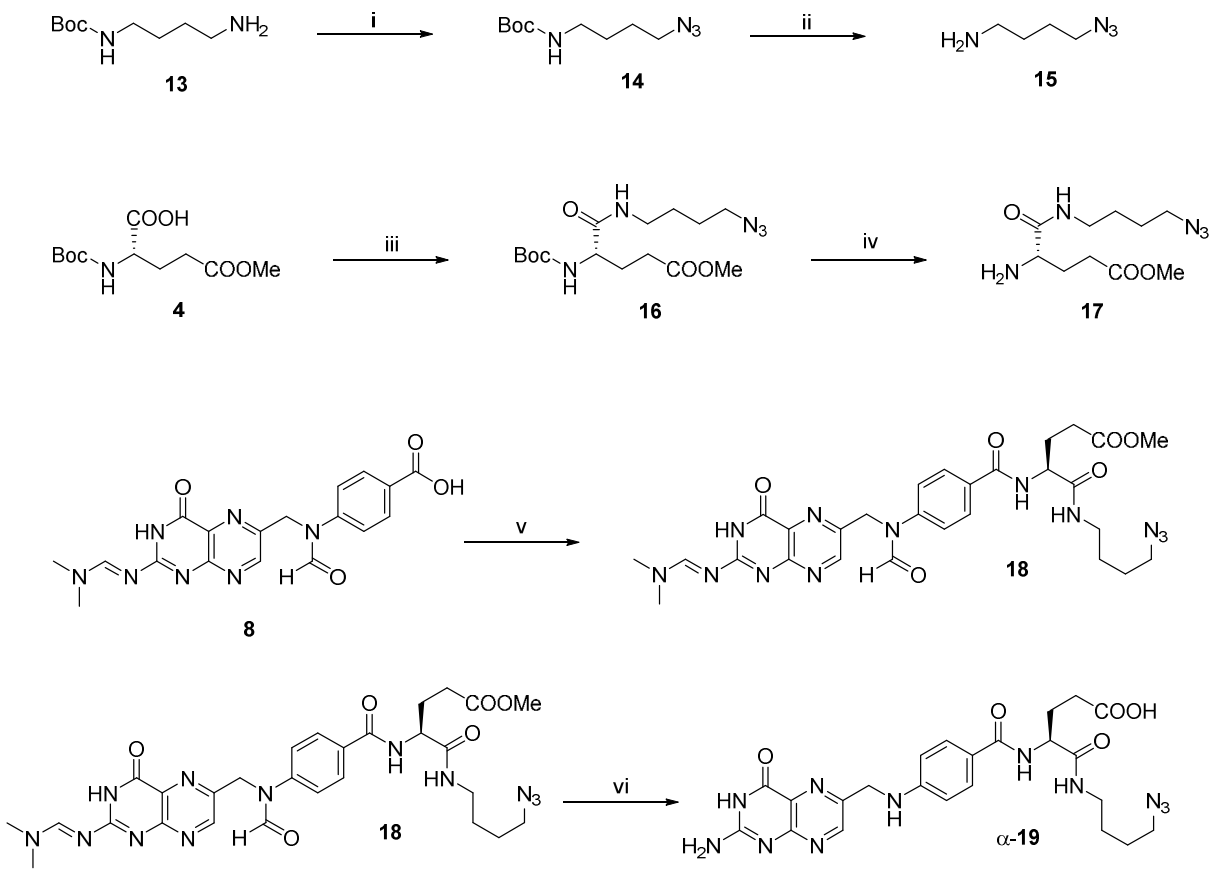

[a](i) 2-Fluoroethyl azide **12**, $Cu(OAc)_2 \times H_2O$, Na-(+)-*L*-ascorbate, MeCN/$H_2O$/EtOH, 60 min, rt; α-**2**: 29%, γ-**2**: 34%.

**Synthesis of α- and γ-Azide Folate Isomers.** The synthesis of the α-azide folate (α-**19**, Scheme 4) was accomplished following the procedure described previously for the γ-regioisomer (γ-**19**).[14] A solution of $NaN_3$ in $CH_2Cl_2/H_2O$ (2:1, 15 mL) was slowly treated with $Tf_2O$ at 0 °C under vigorous stirring. The solution was allowed to warm to room temperature and stirred for 2h. Extraction of the $TfN_3$ was performed with $CH_2Cl_2$ (2×5 mL). Thereafter, the combined organic phases were washed with saturated $Na_2CO_3$ (15 mL) and then slowly added to a solution of *N*-1-Boc-1,4-diaminobutane·HCl (500 mg, 2.22 mmol), $K_2CO_3$ (460 mg, 3.33 mmol) and $CuSO_4 \times 5H_2O$ (5 mg, 0.02 mmol) in MeOH/$H_2O$ (3:2, 25 mL) and stirred at room temperature overnight. The organic solvents were evaporated and the pH of the solution was adjusted to pH 6 by the addition of concentrated HCl. Dilution of the reaction solution was performed with 0.2 M phosphate buffer pH 6.2 (15 mL) and an extraction with $CH_2Cl_2$ (4×40 mL) was performed. The combined organic phases were washed with brine, dried over $MgSO_4$ and the concentrated under reduced pressure to give compound **14** as a colorless oil (339 mg, 96 %). Boc deprotection was

performed by dissolving intermediate **14** (214 mg, 1.0 mmol) in $CH_2Cl_2$/TFA (9:1, 5 mL) and stirring at room temperature overnight. The organic solvent was evaporated and the obtained oily product was directly used for the following coupling with the protected glutamate. For the coupling, a solution of Boc-Glu(OMe)-OH×DCHA **4** (443 mg, 1.0 mmol) in DMF (5 mL) was treated with DIPEA (510 µL, 3.0 mmol) at 0 °C under stirring. HBTU (380 mg, 1.0 mmol) was added and the solution was stirred at 0 °C for 30 min. Intermediate **15** was dissolved in DMF (5 mL) and DIPEA (255 µL, 1.5 mmol) and thereafter the solution was slowly added to the other solution containing the glutamate and stirred at 0 °C for 2 h. Stirring was continued overnight at room temperature. Citric acid (1M, 75 mL) was added to the brownish transparent solution and an extraction with EtOAc (4×40 mL) was carried out. The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated to give the crude mixture of the product. Purification was achieved by flash chromatography on silica gel with hexane/EtOAc (1:2), providing the desired product **16** as a transparent oil (230 mg, 64 %). Boc deprotection was achieved by dissolving **16** in $CH_2Cl_2$/TFA (9:1, 5 mL) and stirring at room temperature until TLC showed completion of the reaction. The organic solvents were evaporated and the obtained product **17** was directly used for the coupling reaction without any further purification. For the coupling, a solution of $N^2$-N,N-dimethylaminomethylen-10-formyl-pteroic acid **8** (233 mg, 0.59 mmol) in DMF (10 mL) at 0 °C was treated with TEA (124 µL, 0.9 mmol). HBTU (224, 0.59 mmol) was added and the solution was stirred at 0 °C for 1 h. Afterwards, a solution containing **17** (220 mg, 0.59 mmol) in DMF (9 mL) and TEA (295 µL, 2.1 mmol) was slowly added and stirred at 0 °C for 1 h. Stirring was continued overnight at room temperature. $H_2O$ (100 mL) was added to the solution and an extraction with EtOAc (2×50 mL) and $CH_2Cl_2$ (2×50 mL) was performed. The combined organic phases were washed with brine, dried over $MgSO_4$ and

concentrated. Flash chromatography on silica gel with $CH_2Cl_2$/MeOH (7 → 9% of MeOH) and evaporation of the solvent gave **18** in 55% yield (204 mg). For deprotection, **18** (108 mg, 0.17 mmol) was dissolved in 1M NaOH (2.5 mL) and the solution was stirred overnight at room temperature. Precipitation of product α-**19** was achieved by acidification to pH 2 using concentrated HCl. The solid could be separated by centrifugation (3500 rpm, 10 min), after which the precipitation was dissolved in 10 mM $NH_4HCO_3$ and purified *via* preparative HPLC using 10 mM $NH_4HCO_3$ solution (solvent A) and MeCN (solvent B) as solvent system and a gradient as follows: 0-50 min: 100-75% A, 50-80 min 75% A, 80-90 min 75-50% A, 90-91 min 50-100% A, 91-100 min: 100% A. After preparative HPLC, the α- and γ-azide folates α-**19** and γ-**19** were obtained in an overall chemical yield of 21% and 25%, respectively. Chemical purity was greater than 95%.

**Scheme 4.** Synthesis of α-folate azide isomer[a]



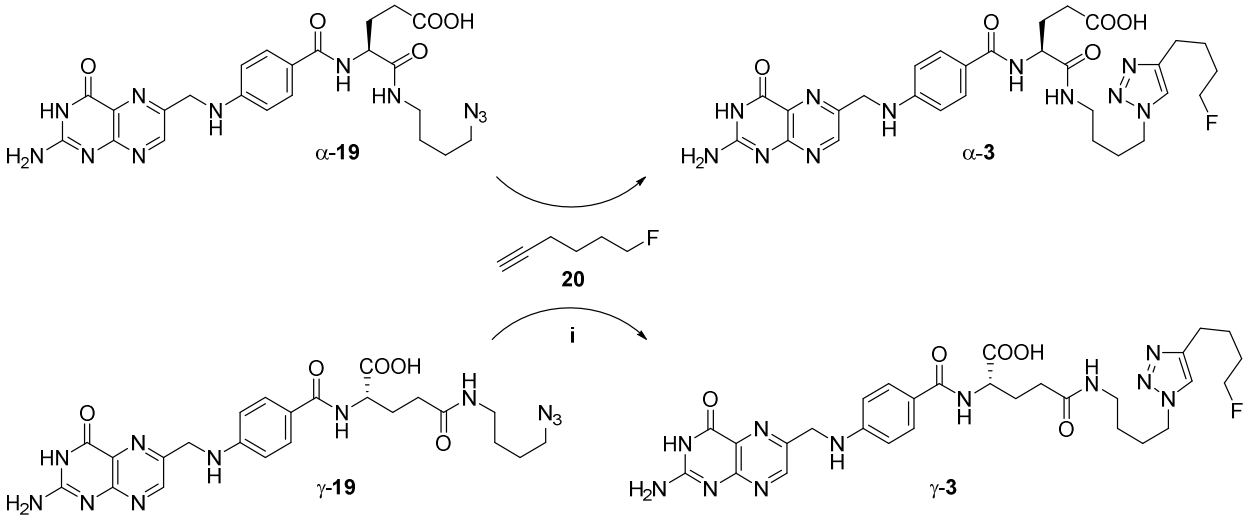
[a](i) 1. $Tf_2O$, $NaN_3$, $CH_2Cl_2/H_2O$, 0 °C to rt, 2h; 2. $TfN_3$, $K_2CO_3$, $CuSO_4 \times 5H_2O$, MeOH/$H_2O$, rt, 20 h, 96%; (ii) $CH_2Cl_2$/TFA (9:1, 5 mL), rt, 16 h; (iii) **15**, HBTU, DIPEA, DMF, 0 °C to rt, 18 h, 64%; (iv) $CH_2Cl_2$/TFA (9:1, 5 mL), rt, 5 h, quant.; (v) **17**, HBTU, TEA, DMF, 0 °C to rt, 24 h, 55%; (vi) 1 M NaOH, rt, 15 h, 63%.

**Synthesis of 6-Fluorohex-1-yne.** Compound **20** was prepared according to a standard procedure published in the literature (Scheme S3, Supporting Information).[14]

**Synthesis of α- and γ-Click-FB Folates.** The α- and γ-click-FB folates (α/γ-**3**) were synthesized following the procedure previously described for the γ-regioisomers.[14] The synthesis of the α-click-FB folate followed the same synthetic procedure (Scheme 5). α-Folate azide α-**19** (5 mg,

0.01 mmol) was added to the solution of **20** (10 mg, 0.1 mmol) in *tert*-amyl alcohol. Aqueous solutions of $Cu(OAc)_2 \times H_2O$ (100 µL, 4 mM) and Na-(+)-*L*-ascorbate (200 µL, 4 mM) were added and stirred at 75 °C for 2h. Reaction control was performed by analytical HPLC. After completion of the reaction, semi-preparative purification was performed using 10 mM $NH_4HCO_3$ (solvent A) and MeCN (solvent B) as a solvent system with a gradient from 0-2 min 100% A, 2-20 min 100-70% A, 20-35 min 70-20% A, 35-40 min 20% A, 40-41 min 20-100% A, 41-45 min 100% A. Reference compounds α-**3** and γ-**3** were obtained after semipreparative HPLC in 65% (4.2 mg) and 81% (0.96 mg) yield, respectively, and high chemical purities of >99%. α-**3**: $^1$H NMR (DMSO-$d_6$) δ/ppm 9.40 (m, 1H), 8.49 (d, $^3J$ = 7.2 Hz, 1H), 7.84 (s, 2H), 7.67 (d, $^3J$ = 8.0 Hz, 2H), 7.31 (s, 2H), 6.80 (s, 1H), 6.58 (d, $^3J$ = 8.0 Hz, 2H), 4.48 (t, $^3J$ = 7.0 Hz, 1H), 4.41 – 4.38 (m, 1H), 4.34 (bs, 2H), 4.28 (t, $^3J$ = 7.0 Hz, 2H), 4.08 (bs, 1H), 3.08 – 3.02 (m, 2H), 2.61 (t, $^3J$ = 7.0 Hz, 2H), 2.19 – 2.11 (m, 1H), 2.09 – 2.00 (m, 1H), 1.93 – 1.80 (m, 2H), 1.79 – 1.72 (m, 2H), 1.65 (m, 4H), 1.33 (m, 2H), 1.23 (s, 2H); HR-MS ($ES^+$) calculated for $C_{29}H_{37}FN_{11}O_5$: 638.2956; found: 638.2957.

**Scheme 5.** Synthesis of reference compounds α- and γ-click-FB folate[a]



[a](i) **26**, $Cu(OAc)_2 \times H_2O$, Na-(+)-*L*-ascorbate, *tert*-amyl alcohol/$H_2O$, 75 °C, 2 h; α-**3**: 65%; γ-**3**: 81%.

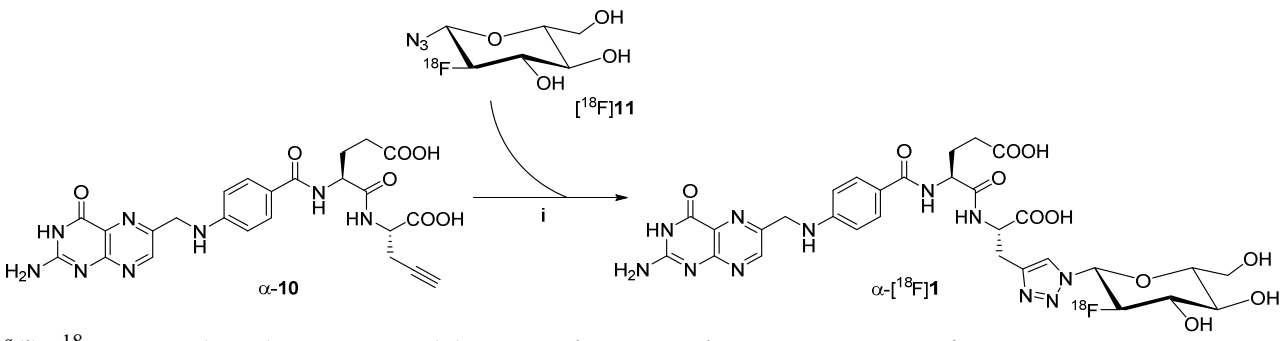
**Radiochemistry**

**Production of Dried [$^{18}$F]Fluoride.** The procedure for the production of dried [$^{18}$F]fluoride is available in the Supporting Information.

**Preparation of 2-deoxy-2-[$^{18}$F]fluoropyranosylazide.** 2-deoxy-2-[$^{18}$F]fluoropyranosylazide ([$^{18}$F]**11**) was prepared as previously reported in the literature (Scheme S4, Supporting Information).[12,13]

**Preparation of α- and γ-Click-[$^{18}$F]FDG Folates**. α- and γ-click-[$^{18}$F]FDG folates (α/γ-[$^{18}$F]**1**) were prepared as previously described (Scheme 6).[12] For α-click-FDG folate (α-[$^{18}$F]**1**), the neutralized 2-deoxy-2-[$^{18}$F]fluoropyranosylazide ([$^{18}$F]**11**) solution was added to α-folate alkyne α-**10** (2 mg, 2.14 mmol) in anhydrous DMF (0.4 mL), containing an aqueous solution of $Cu(OAc)_2 \times H_2O$ (20 μL, 50 mM) and Na-(+)-*L*-ascorbate (40 μL, 50 mM). After stirring the reaction mixture for 15 min at 50 °C phosphate buffered saline (PBS, 0.15 M, 3 mL) was added. The desired product was purified by semipreparative radio-HPLC and the product fraction containing α-click-[$^{18}$F]FDG folate was collected and passed through a sterile filter. Quality control of the radiolabeled compounds was performed on an analytical radio-HPLC.

**Scheme 6.** Preparation of α-click-[$^{18}$F]FDG folate [a]

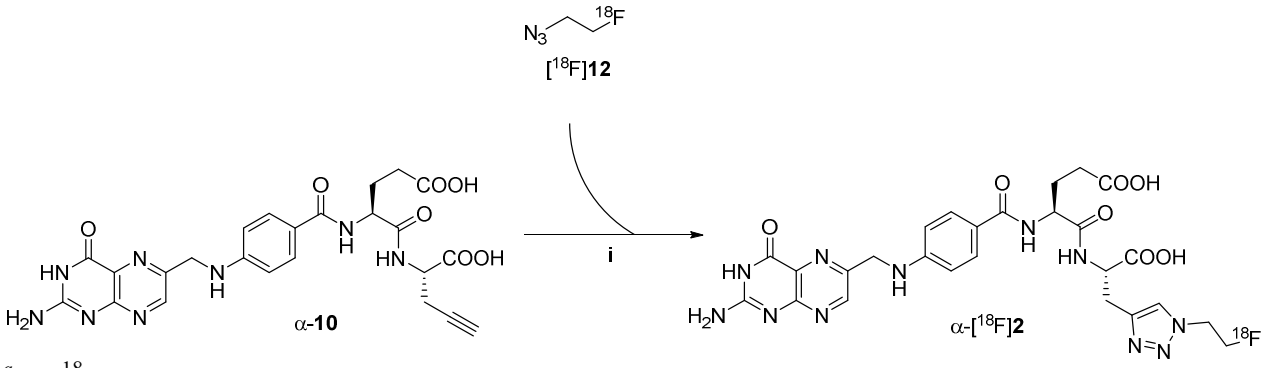



[a](i) [$^{18}$F]**11**, $Cu(OAc)_2 \times H_2O$, Na-(+)-*L*-ascorbate, $H_2O$/DMF, 50 °C, 15 min.

**Preparation of 2-[$^{18}$F]Fluoroethyl azide.** 2-[$^{18}$F]Fluoroethyl azide ([$^{18}$F]**12**) was prepared according a literature procedure (Supporting Information).[15]

**Preparation of α- and γ-Click-[$^{18}$F]FE Folates.** For the syntheses of α-[$^{18}$F]**2** and γ-[$^{18}$F]**2** (Scheme 7), the folate alkyne isomer α/γ-**10** (2 mg, 3.7 μmol) was dissolved in $H_2O$ (500 μL). An ethanolic solution of [$^{18}$F]fluoroethyl azide [$^{18}$F]**12** was passed through a Sep-Pak light Silica cartridge (Waters, preconditioned with 5 mL $Et_2O$) into the reactivial, which contained the dissolved folate alkyne. Additional EtOH (400 μL) was added to the reactivial of 2-[$^{18}$F]fluoroethyl azide reaction and then also passed through the cartridge into the folate alkyne reactivial. A solution of $Cu(OAc)_2 \times H_2O$ in $H_2O$ (100 mM, 50 μL) and Na-(+)-*L*-ascorbate in $H_2O$ (100 mM, 100 μL) were added and the resulting mixture was heated at 80 °C for 20 min. Then, the reaction solution was allowed to cool down for 5 min and sodium phosphate buffer (20 mM, pH 7.4, 3.5 mL) was added. Purification was carried out with a semipreparative radio-HPLC and the product fraction was collected. Quality control of the radiolabeled compounds were performed on an analytical radio-HPLC.

**Scheme 7.** Two-step radiosynthesis of α-click-[$^{18}$F]FE folate (α-[$^{18}$F]**2**) [a]



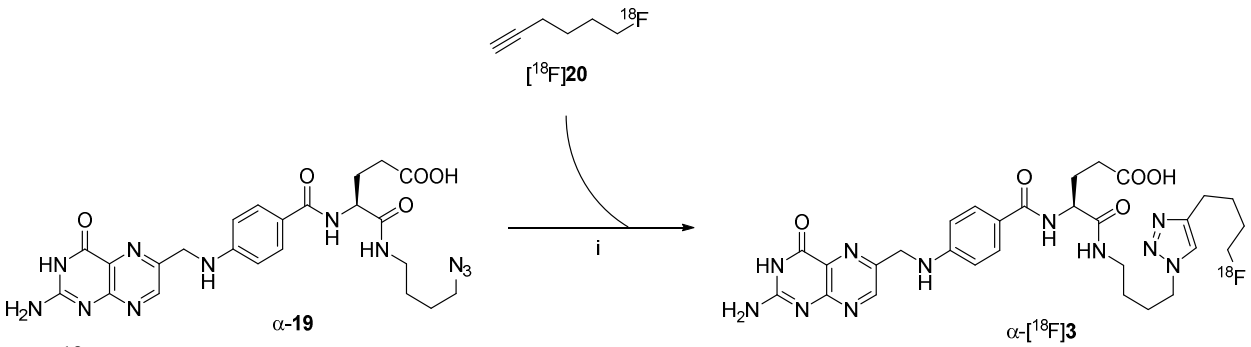

[a](i) [$^{18}$F]**12**, Cu(OAc)$_2$×H$_2$O, Na-(+)-*L*-ascorbate, EtOH/H$_2$O/MeCN, 80 °C, 20 min.

**Preparation of 6-[$^{18}$F]Fluorohex-1-yne.** 6-[$^{18}$F]Fluorohex-1-yne ([$^{18}$F]**20**) was prepared in analogy to previously reported methods (Supporting Information).[14, 15]

**Preparation of α- and γ-Click-[$^{18}$F]FB Folates**. Folate azide α-**19** (1 mg, 1.86 μmol) (Scheme 8) was dissolved in DMF (230 μL) and DMSO (70 μL) in a reactivial (5 mL). Afterwards, 6-[$^{18}$F]fluorohex-1-yne [$^{18}$F]**20** in MeCN (200 μL) was distilled in the reactivial containing the dissolved folate azide. Additional MeCN (200 μL) was added to the reactivial of the 6-[$^{18}$F]fluorohex-1-yne reaction and also distilled into the reactivial containing the folate azide. Aqueous solutions of CuSO$_4$×5H$_2$O (35 mM, 100 μL) and Na-(+)-*L*-ascorbate (70 mM, 100 μL) were added and the resulting mixture was heated at 90 °C for 20 min. The reaction solution was allowed to cool down for 5 min and diluted with sodium phosphate buffer (20 mM, pH 7.4, 3.5 mL). Purification was carried out with a semipreparative radio-HPLC. After collection of the product in a penicillin vial containing H$_2$O (40 mL), the diluted product solution was transferred twice through two Oasis MCX 1 cc cartridges (Waters, preconditioned with 5 mL EtOH, followed by 10 mL H$_2$O) connected in series. After washing of the MCX cartridges with H$_2$O (3 mL), the product was eluted with a solution of 20% EtOH in 50 mM sodium phosphate buffer

pH 7.4 (1.5 mL). Dilution of the product solution with aqueous 0.9% NaCl solution (4.5 mL) gave a final EtOH concentration of 5%. In the last step, the product solution was passed through a sterile filter. Quality control of the radiolabeled compounds was performed on an analytical radio-HPLC.

**Scheme 8.** Two-step radiosynthesis of α-click-[$^{18}$F]FB folate (α-[$^{18}$F]**3**)[a]




[a](i) [$^{18}$F]**20**, $CuSO_4 \times 5H_2O$, Na-(+)-*L*-ascorbate, DMSO/DMF/$H_2O$/MeCN, 90 °C, 20 min.

**Determination of Distribution Coefficient.** The distribution coefficients ($logD_{7.4}$) of α/γ-[$^{18}$F]**1**, α/γ-[$^{18}$F]**2** and α/γ-[$^{18}$F]**3** were determined using the shake flask method.[16] Briefly, the radiotracer was dissolved in a mixture of phosphate buffer (500 µL, pH 7.4) and *n*-octanol (500 µL) at room temperature. The sample was shaken for 15 min at room temperature in an over-head shaker and afterwards centrifuged (3 min, 5000 rpm) to separate the two phases. Aliquots (50 µL) each of both phases were analyzed in a γ-counter (Wizard, PerkinElmer). The partition coefficient is expressed as the ratio between the radioactivity concentrations (cpm/ml) of the *n*-octanol and the buffer phase. Values represent the mean ± standard deviation of sextuples from two independent experiments.

**In Vitro Binding Affinity.** The binding affinity of reference compounds α/γ-**1**, α/γ-**2** and α/γ-**3** were tested in a competitive *in vitro* binding assay on FR-positive KB cells according to a previously published procedure (Figure S4, Supporting Information).[27]

**Animals.** Animal experiments were performed in compliance with Swiss and local laws on animal protection and approved by the Veterinary Office of Switzerland. Female CD-1 nude mice were purchased from Charles River (Germany) and kept on a folate-deficient rodent diet (Harlan Laboratories, US) for 3 weeks to reduce blood-folate levels as previously reported.[28] Animals were inoculated subcutaneously with a KB tumor cell suspension ($5\times10^6$ cells in 0.1 mL PBS pH 7.4) on both shoulders of each mouse. After 1-2 weeks KB tumor-bearing mice were used for biodistribution studies and PET/CT scans.

**Biodistribution Studies**. Animals were injected with ~5 MBq (~0.1 nmol, 100 µL) of the corresponding radiotracer *via* a lateral tail vein ($n$ = 4 per time point). Blocking studies ($n$ = 3) were performed with excess folic acid dissolved in PBS pH 7.4 (1 mg/mL) which was intravenously injected (100 µL per mouse) immediately before the radiotracer injection. Animals were sacrificed at pre-defined time points. Organs and tissues were collected and measured in a γ-counter. The incorporated radioactivity was expressed as percentage of injected activity per gram of tissue [% IA/g].

**PET/CT Imaging Studies.** PET/CT experiments were performed with an eXplore VISTA PET/CT tomograph (Sedecal, Madrid, Spain). Animals received 11-22 MBq (~0.1-0.3 nmol) of the corresponding radiotracer (100−200 µL per injection) *via* lateral tail vein injection ($n$ = 1).

For blocking studies ($n$ = 1), mice were injected with excess folic acid dissolved in PBS pH 7.4 (1 mg/mL) which was intravenously injected (100 μg in 100 μL) prior to the radiotracer injection. Animals were anesthetized with isoflurane in an air/oxygen mixture and scanned as described previously.[29] Whole body-PET scans were acquired from 75-105 min p.i. followed by a CT. After acquisition, PET data were reconstructed in user-defined time frames. The fused data sets of PET and CT were analyzed with PMOD post-processing software (version 3.6, PMOD Technologies Ltd., Zurich, Switzerland). Tissue radioactivity was expressed as standardized uptake values (SUV), which is the decay-corrected radioactivity per $cm^3$ divided by the injected radioactivity dose per gram (IA/g) of body weight.

**Metabolite Studies.** For the determination of radiometabolites, the folate radiotracers (50-97 MBq; ~1.2-2.3 nmol) were intravenously injected into mice. Mice were sacrificed 30 min after radiotracer injection and blood, urine, liver and tumor were collected and analyzed. Blood samples were centrifuged at 5000 g for 5 min at 4 °C, followed by precipitation of the proteins of the plasma samples by addition of ice-cold methanol. After centrifugation, the supernatants of the plasma samples and the urine samples were analyzed by radio-UPLC, respectively. Tumor tissue was homogenized in an equal volume of PBS using a PT 1200 C Polytron (Kinematica AG) and centrifuged at 5000 g for 5 min at 4 °C. Ice-cold methanol was added to the supernatant to precipitate the proteins. After centrifugation, the supernatant was analyzed by radio-UPLC.

## ASSOCIATED CONTENT

## Supporting Information

Experimental details, (radio-)HPLC methods, *in vivo* metabolism data, binding curves, and biodistribution data. This material is available free of charge via the Internet at http://pubs.acs.org

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### Notes

The authors declare no competing financial interest.

## ACKNOWLEDGMENTS

We thank Claudia Keller, Dr. Adrienne Herde, Raffaella M. Schmid, Nadja Romano, Klaudia Siwowska and Bianca Bochsler for their support and technical assistance. This work was financially supported by Merck & Cie (Schaffhausen, Switzerland) and by the Swiss National Science Foundation.

## ABBREVIATIONS

FR – folate receptor; click-FDG folate – click-fluoro-deoxy-glucose folate; click-FE folate – click-fluoroethyl folate; click-FB folate – click-fluorobutyl folate; RCY – radiochemical yield; SA – specific activity; PCFT – proton-coupled folate transporter

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