Synthesis of Precursors for ^{18}F-Labeling of Folic Acid for PET Application

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Received 14 July 2011; revised 24 August 2011

Abstract: Radiolabeled folate derivatives have the potential to target folate receptor-positive tumor cells for noninvasive diagnosis via positron emission tomography (PET) and single photon emission computed tomography (SPECT). We report the regiospecific synthesis of N^2-(*N*,*N*-dimethylaminomethylene)-2′-nitrofolic acid di-*tert*-butyl ester (**13**) and N^2-(*N*,*N*-dimethylaminomethylene)-N^{10}-formyl-2′-nitrofolic acid dimethyl ester (**25**), which are precursors for the radiolabeling of folic acid with the PET isotope ^{18}F. A modular synthetic strategy was applied: Fmoc- and Boc-protected 4-amino-2-nitrobenzoic acid were linked via amide bonds to di-*tert*-butyl L-glutamate and dimethyl L-glutamate, respectively, to form building blocks **10** and **19**. After Fmoc and Boc removal, both compounds were coupled to 6-(bromomethyl)pterin hydrobromide to give crude 2′-nitrofolic acid di-*tert*-butyl ester and 2′-nitrofolic acid dimethyl ester. After formylation of 2′-nitrofolic acid dimethyl ester at N^{10} and the introduction of an *N*,*N*-dimethylaminomethylene group at N^2, precursor **25** was obtained in an overall yield of 3%. The analogous 2′-fluoroderivative **28** was obtained in 7% overall yield from 4-amino-2-fluorobenzoic acid. Precursor **13** was obtained from 2′-nitrofolic acid di-*tert*-butyl ester in 6% yield after N^2-protection. The synthesis of the reference materials 2′-nitro- and 2′-fluorofolic acid was achieved by the reaction of *N*-(4-amino-2-nitrobenzoyl)- and *N*-(4-amino-2-fluorobenzoyl)-L-glutamic acid with 6-(bromomethyl)pterin hydrobromide, giving 7% and 14% overall yield, respectively.

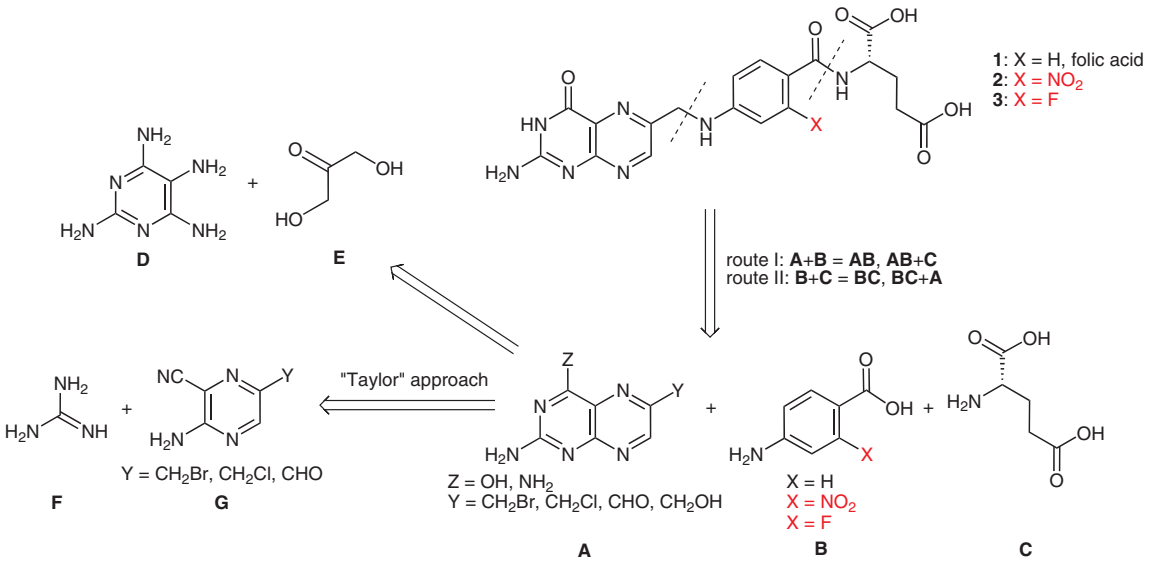
Key words: nucleophilic substitution, halides, amino acids, protecting groups

The folate receptor is a promising tumor target for folate conjugates since it is overexpressed in a wide variety of cancer cells.[2] A number of folate conjugates have been synthesized for application in tumor therapy and diagnosis[3] (e.g., chemotherapeutic agents,[4] antisense oligonucleotides,[5] antibodies,[6] protein toxins,[7] and liposomes[8]). Several folate-based radiopharmaceuticals have been developed and labeled with radionuclides including $^{66/67/68}$Ga,[9] ^{99m}Tc,[10] and ^{111}In[11] for diagnostic application using molecular imaging methods such as positron emission tomography (PET) and single photon emission computed tomography (SPECT). Folate derivatives suitable for labeling with the PET radionuclide ^{18}F are especially attractive since this isotope exhibits excellent decay properties [half-life: 110 min, β^+-E_{av}: 250 keV (97%)] for tumor diagnosis by PET. There are only a few folate-based PET tracers reported in the literature where a prosthetic group bearing an ^{18}F label is conjugated to either of the two carboxyl groups of folic acid (**1**).[12] In contrast to the published methods that utilize prosthetic groups, we have decided on an approach that incorporates the ^{18}F-label directly into the benzoyl moiety of folic acid by a formal bioisosteric replacement of a phenyl hydrogen atom. Since the 'direct labeling' approach involves only a slight structural modification of folic acid, we expect the labeled compound to mimic the folic acid most closely and consequently to retain a high affinity for the folate receptor and a metabolic pathway similar to that of folic acid.

In order to obtain a sufficiently high level of radioactivity for subsequent in vivo studies, the incorporation of the ^{18}F-label is usually carried out during the last step of a multi-step synthesis. Nucleophilic substitution at a phenyl ring, afforded by leaving and activating groups, is one of the standard procedures for the introduction of the ^{18}F-label.[13] The 2′-position in the benzoyl moiety of folic acid is activated by a carboxylamide, and we therefore initially envisaged the incorporation of the ^{18}F-label in the 2′-position by exchange of a nitro group. Since the nitration of folic acid leads to a 3′,5′-dinitro derivative[14] and protection of the NH_2 group and the carboxyl groups of folic acid is necessary during ^{18}F-labeling, we developed a modular synthesis for N^2-(*N*,*N*-dimethylaminomethylene)-2′-nitrofolic acid di-*tert*-butyl ester (**13**) and N^2-(*N*,*N*-dimethylaminomethylene)-N^{10}-formyl-2′-nitrofolic acid dimethyl ester (**25**), which were used as precursors for the synthesis of 2′-[^{18}F]fluorofolic acid (**29**). We describe here the syntheses of precursors **13** and **25** as well as the reference compounds 2′-nitrofolic acid (**2**), 2′-fluorofolic acid (**3**),[15] and N^2-(*N*,*N*-dimethylaminomethylene)-2′-fluoro-N^{10}-formylfolic acid dimethyl ester (**28**). The ^{18}F-labeling of the precursors and the preclinical evaluation of 2′-[^{18}F]fluorofolic acid are described elsewhere.[16]

In general, there are two different routes for the regiospecific synthesis of folic acid (**1**) that is functionalized in the 4-aminobenzoyl moiety (Scheme 1). Route I comprises the coupling of synthons **A** and **B** to give a 2′-functionalized derivative of pteroic acid (**AB**), which is conjugated with L-glutamic acid (synthon **C**) to give **2** and **3**. Route II

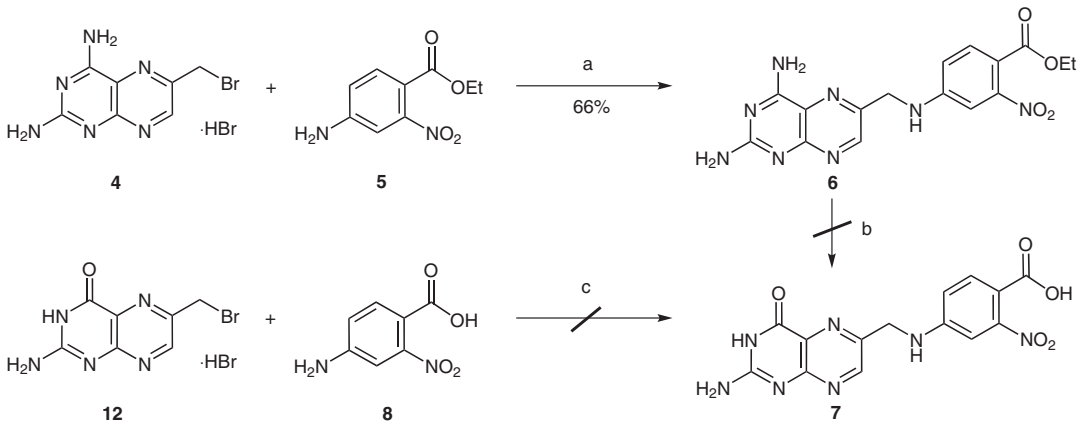
SYNTHESIS 2011, No. 22, pp 3639–3648
Advanced online publication: 29.09.2011
DOI: 10.1055/s-0030-1260247; Art ID: T69811SS



Scheme 1 Retrosynthesis for folic acid (**1**) and 2′-functionalized folic acid derivatives **2** and **3**

includes the synthesis of 2′-functionalized 4-aminobenzoyl-L-glutamic acid by reaction of **B** with **C** and finally coupling to synthon **A**. The key step of the Taylor approach[17] comprises the condensation of a 5-substituted 2-amino-3-cyanopyrazine **G**[18] with guanidine **F** to give a 6-substituted 4-aminopteridine. If **G** is 5-substituted with a methylaminobenzoic acid moiety, the resulting synthon **GB** gives, after condensation with **F**, a derivative of pteroic acid (**AB**). Since the L-glutamic acid moiety extensively racemizes during the ring closure reaction with guanidine,[19] the Taylor approach is only applicable to synthesis of synthon **A** (**F** + **G**) and pteroic acid (**F** + **GB**). However, since Bader et al.[20] described the dialkylation of primary amines by 2-amino-5-chloromethyl-3-cyanopyrazine, we decided not to follow the Taylor approach. Instead, we started with synthon **A**, which was obtained by condensation of 2,4,5,6-tetraaminopyrimidine (**D**) with dihydroxyacetone (**E**) according to Baugh et al.[21] and subsequent derivatization of the intermediate 6-hydroxymethylpteridine derivative.

The structure of **A** is variable and determines the method of coupling. If **A** is a 6-formyl derivative[22] (Y = CHO), coupling with **B** or **BC** is achieved by reductive amination,[23] whereas the 6-bromomethyl derivative[24] (Y = CH_2Br) is coupled by alkylation of the amine **B** or **BC**.[25] Since the reductive amination may affect the nitro group of synthon **B** or **BC**, the investigations were started with the bromomethyl derivative **4**[24,25] (Scheme 2). Following route I and by analogy to Montgomery and coworkers,[25] 4-amino-2-nitrobenzoic acid ethyl ester[26] (**5**) was coupled to 2,4-diamino-6-(bromomethyl)pteridine hydrobromide (**4**) in *N*,*N*-dimethylacetamide (DMAC) at 60 °C to give ethyl 4-[(2,4-diaminopteridine-6-yl)methylamino]-2′-nitrobenzoate (**6**) in 66% yield. Simultaneous hydrolysis of the 4-amino-function and ethyl ester moiety of **6** with aqueous sodium or lithium hydroxide under anaerobic conditions by analogy to Seeger et al.[27] or aqueous hydrochloric acid[21,28] to give 2′-nitropteroic acid (**7**) failed. On the other hand, fast and complete degradation of starting material was observed under alkaline condi-



Scheme 2 Synthesis of ethyl 4-[(2,4-diaminopteridine-6-yl)methylamino]-2′-nitrobenzoate (**6**) following route I. *Reagents and conditions*: (a) DMAC, 60 °C, 3 h; (b) 0.1 M or 2 M or 4 M aq NaOH, r.t. to –80 °C or LiOH (3 equiv) in H_2O–MeOH (1:1) or 1 M or 6 M aq HCl, r.t. to –100 °C or HBr, 80–95 °C; (c) DMF, 100 °C, 5 h.

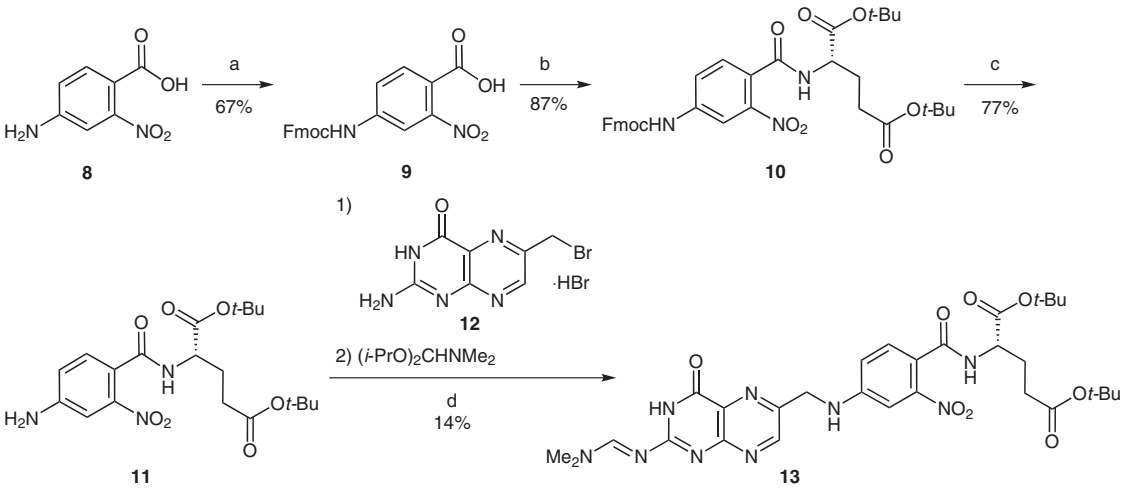
tions, whereas with hydrochloric acid no conversion took place, even at 100 °C or after the addition of methanol to increase solubility of the starting material. While the use of 48% hydrobromic acid at 95 °C mainly led to formation of an unknown by-product, the hydrolysis with 62% hydrobromic acid at 80 °C gave a mixture of different compounds including the desired product **7** with up to 44% area detected by LC-MS. Due to poor solubility of the product, isolation and purification of the latter was impossible. In order to circumvent the hydrolysis of the 4-amino and ethyl ester moieties, the coupling of 4-amino-2-nitrobenzoic acid (**8**) with 6-(bromomethyl)pterin hydrobromide (**12**) to obtain 2′-nitropteroic acid (**7**) was then investigated (Scheme 2). The crude product was obtained only in poor yield and purity. Purification of the latter by dissolving it in aqueous sodium hydroxide, and subsequent treatment with charcoal and precipitation by adding acetic acid did not improve purity, which was approximately 47% area determined by LC-MS. Formylation of the crude material at N^{10} with formic acid and subsequent treatment with *N*,*N*-dimethylformamide diisopropylacetal did not lead to a uniform product that could be purified. The synthesis of 2′-nitropteroic acid (**7**) as intermediate for the synthesis of **2** was therefore abandoned.

Following route II, 4-amino-2-nitrobenzoic acid (**8**) was protected with 9-fluorenylmethoxycarbonyl (Fmoc) in 67% yield (Scheme 3). The 4-(*N*-Fmoc-amino)-2-nitrobenzoic acid (**9**) was coupled to L-glutamic acid di-*tert*-butyl ester in 87% yield using 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/triethylamine in DMF. Fmoc deprotection was achieved by a standard procedure[29] to give *N*-(4-amino-2-nitrobenzoyl)-L-glutamic acid di-*tert*-butyl ester (**11**) in a yield of 77%. Compound **11** was coupled to 6-(bromomethyl)pterin hydrobromide[24,25] (**12**) in DMAC at 60 °C to give crude 2′-nitrofolic acid di-*tert*-butyl ester. Since the latter was poorly soluble in organic solvents it was not further purified, but was converted directly into N^2-(*N*,*N*-dimethylaminomethylene)-2′-nitrofolic acid di-*tert*-butyl ester (**13**), which was then purified by flash chromatography on silica gel. Reduced nucleophilicity of the amino functionality of **11** caused by the electron-withdrawing nitro substituent in position 2 and repeated chromatographic purification of **13** resulted in only a poor yield of 14% for **13** over the last two steps starting from **11**.

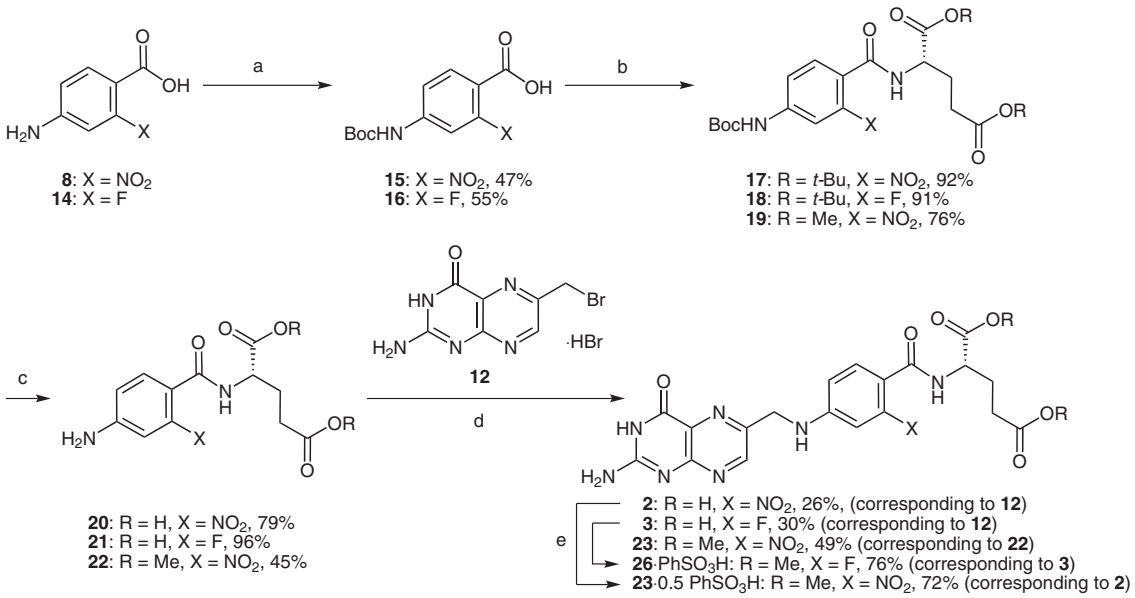
The syntheses of 2′-nitrofolic acid (**2**) and 2′-fluorofolic acid[15] (**3**) were carried out starting from 4-amino-2-nitrobenzoic acid[26] (**8**) and 4-amino-2-fluorobenzoic acid[30] (**14**), respectively, following route II (Schemes 1 and 4). The 4-amino group of **8** and **14** was protected by a *tert*-butoxycarbonyl (Boc) group in 47% and 55% yield, respectively. After coupling **15** and **16** to L-glutamic acid di-*tert*-butyl ester in 92% and 91% yields, using HBTU and Et_3N, global deprotection was achieved by treatment with trifluoroacetic acid to give the trifluoroacetate of *N*-(4-amino-2-nitrobenzoyl)-L-glutamic acid (**20**) in 79% yield and *N*-(4-amino-2-fluorobenzoyl)-L-glutamic acid (**21**) in 96% yield. Coupling of **20** and **21**, respectively, with 6-(bromomethyl)pterin hydrobromide[24,25] (**12**) in DMAC at 60 °C led to 2′-nitrofolic acid (**2**) in 26% yield and 2′-fluorofolic acid (**3**) in 30% yield corresponding to **12**.

The ^{18}F-labeling by nucleophilic substitution of the 2′-nitro group of precursor **13** using [^{18}F]-fluoride cryptate complex, final deprotection with aqueous hydrochloric acid (Scheme 5), and application of the 2′-[^{18}F]fluorofolic acid for PET studies in tumor-bearing mice have been described by Ross et al.[16]

The ^{18}F-labeling of **13** was accomplished with 8% decay-corrected yield. Subsequent deprotection of the labeled product of **13** afforded target compound **29** in 50% yield resulting in an overall radiochemical yield of 4%.[16] To increase the radiochemical yield and prevent decomposition of the fluorinating reagent by hydrogen bond formation, we envisaged additional protection of the N^{10}-amino function by a formyl or acetyl group. The acylation of precursor **13** or crude 2′-nitrofolic acid di-*tert*-butyl ester was not successful under several nonacidic mild acylation



Scheme 3 Synthesis of N^2-(*N*,*N*-dimethylaminomethylene)-2′-nitrofolic acid di-*tert*-butyl ester (**13**) following route II. *Reagents and conditions*: (a) Fmoc-Cl, Na_2CO_3, H_2O; (b) L-Glu(O-*t*-Bu)O-*t*-Bu, HBTU, Et_3N, DMF; (c) pyrrolidine, DMF; d) 1. DMAC, 60 °C, 3 h, 2. DMF-diisopropylacetal, DMF, r.t., 17 h.

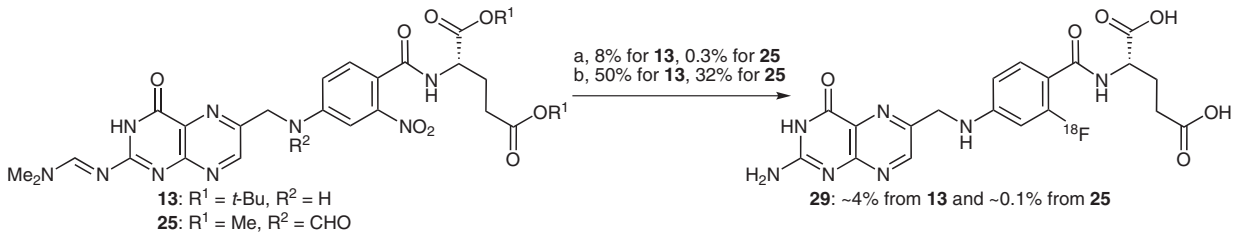


Scheme 4 Synthesis of 2′-nitrofolic acid (**2**), 2′-fluorofolic acid (**3**), and 2′-nitro and 2′-fluorofolic acid dimethyl ester (**23**, **26**). *Reagents and conditions*: (a) Boc_2O, 1,4-dioxane, H_2O, NaOH, r.t.; (b) L-Glu(OR)OR·HCl, HBTU, Et_3N, CH_2Cl_2; (c) TFA, CH_2Cl_2, 0 °C – r.t., 17 h; (d) **12**, DMAC, 60 °C, 6 h; (e) $PhSO_3H$, MeOH, 90 °C, 2 h.

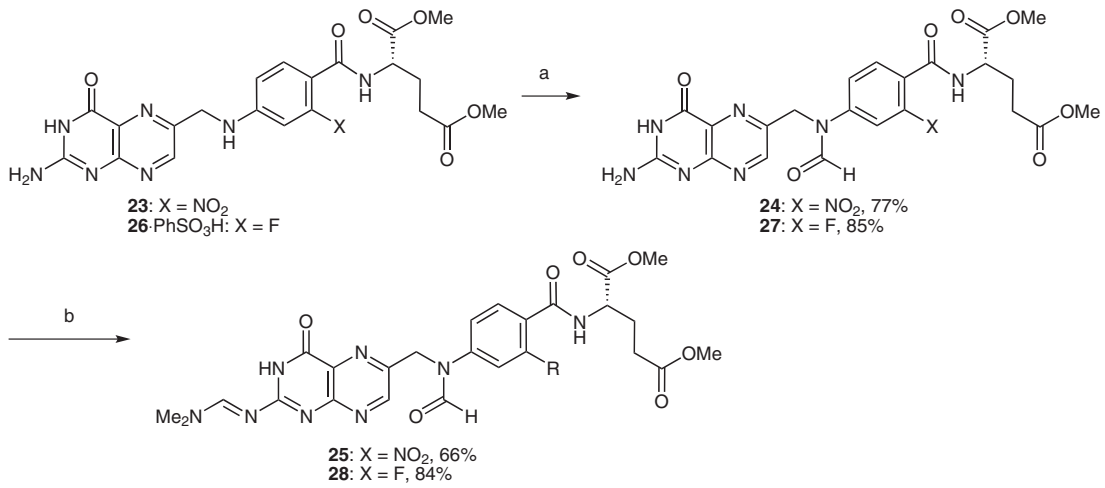
conditions, for example, *p*-nitrophenyl acetate at pH 11 in aqueous sodium hydroxide[31] or 2,4,5-trichlorophenyl formate/*N*,*N*-diisopropylethylamine in *N*,*N*-dimethylformamide[32] or with formic acid activated by *N*,*N*′-dicyclohexylcarbodiimide in pyridine.[33] Either no conversion or an unselective reaction took place, leading to an inseparable mixture of products. We therefore decided to protect the L-glutamic acid moiety with methyl esters instead of acid-labile *tert*-butyl esters. The synthesis of 2′-nitrofolic acid dimethyl ester (**23**) was achieved by two methods. It was done either by esterification of 2′-nitrofolic acid (**2**) in methanol with benzenesulfonic acid (BSA) by analogy to the esterification of folic acid,[34] leading to a hemibenzenesulfonate of 2′-nitrofolic acid dimethyl ester (**23**) in 72% yield, or by the coupling of *N*-(4-amino-2-nitrobenzoyl)-L-glutamic acid dimethyl ester (**22**) to 6-(bromomethyl)pterin hydrobromide (**12**) in 49% yield (Scheme 4). 2′-Nitrofolic acid dimethyl ester (**23**) was then formylated in formic acid in 77% yield to give N^{10}-formyl-2′-nitrofolic acid dimethyl ester (**24**), which was protected at N^2 to give precursor N^2-(*N*,*N*-dimethylaminomethylene)-2′-nitrofolic acid dimethyl ester (**25**) in 66% yield (Scheme 6). The synthesis of N^2-(*N*,*N*-dimethylaminomethylene)-2′-fluorofolic acid dimethyl ester (**28**), which was used as reference material during ^{18}F-labeling, was accomplished in an analogous manner from 2′-fluorofolic acid (**3**), which was esterified with methanol in the presence of benzenesulfonic acid to give 2′-fluorofolic acid dimethyl ester benzenesulfonate (**26**) in 76% yield (Scheme 4). N^{10}-Formylation of **26** occurred in 85% yield, and the introduction of an *N*,*N*-dimethylaminomethylene group at N^2 was carried out in 84% yield to give **28** (Scheme 6).

^{18}F-Labeling of compound **25** (Scheme 5) was accomplished in a yield of only 0.3% due to the low reactivity of the nonactivated aromatic ring. This means that protection of position N^{10} is negligible for improvement of the yield. Deprotection of the labeled compound was effected in 32% yield by using 4 M aqueous hydrochloric acid for 10 minutes at 60 °C and subsequent addition of 5 M aqueous sodium hydroxide solution. This procedure gave 2′-[^{18}F]fluorofolic acid (**29**) in only 0.1% overall yield from precursor **25** under conditions, which were not well optimized. Coinjection of the 2′-[^{18}F]fluorofolic acid (**29**) with the ^{19}F-substituted inactive reference compound **3** proved the detection of the ^{18}F-labeled tracer.[35]

In conclusion, we have identified a synthetic pathway for the preparation of two precursors suitable for the synthesis of 2′-[^{18}F]fluorofolic acid, which is a novel nonconjugated



Scheme 5 Radiosynthesis of 2′-[^{18}F]fluorofolic acid starting from precursors **13** and **25**. *Reagents and conditions*: (a) [^{18}F]-K2.2.2, (b) for **13**: 4 M aq HCl, 12 min, 60 °C; for **25**: 4 M aq HCl, 10 min, 60 °C, then 5 M aq NaOH, 10 min, 60 °C.



Scheme 6 Synthesis of N^2-(*N*,*N*-dimethylaminomethylene)-N^{10}-formyl-2′-nitro- and 2′-fluorofolic acid dimethyl ester (**25**, **28**) from 2′-nitro- and 2′-fluorofolic acid dimethyl ester (**23**, **26**). *Reagents and conditions*: (a) HCO_2H, 60 °C, 3.5 h; (b) DMF-diisopropylacetal, DMF, r.t., 17 h.

PET tracer of folic acid. Preliminary in vitro and in vivo experiments performed with folate receptor-positive cells in culture and folate receptor-positive tumor-bearing mice have demonstrated the superior characteristics of the novel ^{18}F-folate PET tracer compared to previously published ^{18}F-folate PET tracers prepared with ^{18}F-labeled prosthetic groups. However, the overall radiochemical yields will significantly limit the use of the precursors for the routine preparation of 2′-[^{18}F]fluorofolic acid for clinical applications. In order to improve the radiochemical yield we are currently investigating the synthesis of precursors in which a hydrogen atom of one of the methylene groups in the glutamic acid moiety of folic acid is replaced by a sulfonic acid ester. Aliphatic sulfonic acid esters can be smoothly modified by nucleophilic ^{18}F-substitution.[36] Presumably this approach will allow an efficient one-step direct labeling of folic acid with ^{18}F.

All experiments were carried out under an argon or N_2 atmosphere. All commercially available reagents and solvents were used as received. Methyl *tert*-butyl ether is abbreviated as MTBE. The N^2-(*N*,*N*-dimethylaminomethylene)-10-formylpteroic acid (**11**) was provided by Merck & Cie, Schaffhausen, Switzerland. 4-Amino-2-nitrobenzoic acid[26] (**8**) and the corresponding ethyl ester[26] (**5**) were purchased from Chemie Brunschwig AG. 4-Amino-2-fluorobenzoic acid[30] (**14**), *N*,*N*-dimethylformamide diisopropylacetal and dimethyl-L-glutamate hydrochloride were purchased from Sigma Aldrich. Di-*tert*-butyl-L-glutamate hydrochloride was purchased from Novabiochem. TLC was performed on Merck silica gel 60 F254 glass plates and flash column chromatography on Fluka silica gel 60. NMR spectra were recorded on a Mercury Plus 200 MHz spectrometer or on a Bruker Avancell 400 MHz spectrometer, with chemical shifts δ in ppm using TMS or DMSO solvent peak as internal reference. HRMS-ESI were recorded using a Bruker Daltonics maXis ESI-QTOF spectrometer with a resolution of 40000–60000. Four point mass calibration was achieved by using the ESI-L low concentration Tuning Mix G1969-85000 provided by Agilent Technologies. Melting points were determined with a Büchi melting point apparatus B-540 and are uncorrected.

Ethyl 4-[(2,4-Diaminopteridine-6-yl)methylamino]-2′-nitrobenzoate (6)

A mixture of 2,4-diamino-6-(bromomethyl)pteridine hydrobromide[24] (**4**; 7 g, 20.8 mmol) and 4-amino-2-nitrobenzoic acid ethyl ester[26] (**5**; 12.7 g, 60.4 mmol) in DMF (260 mL) was stirred at 100 °C for 5 h. The mixture was cooled to 0 °C, and the product was precipitated by the addition of Et_2O (770 mL). The product was collected by filtration, washed with Et_2O (250 mL), and dried under vacuum at 45 °C to give **6**; yield: 5.3 g (66% corresponding to **4**); greenish-grey powder; mp >250 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.23 (t, ^3J = 7.1 Hz, 3 H, CH_3), 4.21 (q, ^3J = 7.1 Hz, 2 H, CH_2CH_3), 4.71 (s, 2 H, H-6), 7.04 (dd, ^3J = 8.7 Hz, ^4J = 2.2 Hz, 1 H, 5′-H_{arom}), 7.19 (d, ^4J = 2.2 Hz, 1 H, 3′-H_{arom}), 7.73 (d, ^3J = 8.7 Hz, 1 H, 6′-H_{arom}), 8.72, 7.60 (2 br s, 2 H, NH_2), 8.91 (s, 1 H, H-7), 9.37 (br d, 2 H, NH_2).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 13.8, 45.3, 60.9, 106.3, 109.7, 113.8, 121.5, 132.0, 145.2, 149.4, 155.8, 162.7, 163.4.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{16}H_{17}N_8O_4$: 385.1367; found: 385.1372.

4-{[(9*H*-Fluoren-9-yl)methoxy]carbonylamino}-2-nitrobenzoic Acid (9)

9-Fluorenylmethyl chloroformate (17.0 g, 65.7 mmol) in 1,4-dioxane (20 mL) was added dropwise to a solution of 4-amino-2-nitrobenzoic acid[26] (**8**; 11.4 g, 62.6 mmol) in H_2O (228 mL) containing Na_2CO_3 (6.63 g). After stirring for 20 h under N_2, the mixture was filtered, and the filtrate was washed with MTBE (5 × 100 mL). Residual MTBE was removed from the aqueous phase by evaporation under vacuum. Ice-cold H_2O (456 g) was added to the aqueous phase. The mixture was adjusted to pH 3 by the addition of aq 2 M HCl (31 mL). The precipitate was drawn off by suction, washed with H_2O (513 mL), and dried at 40 °C under vacuum to give **9**; yield: 17.0 g (67%); off-white crystals; mp >230 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 4.27 (t, ^3J = 6.3 Hz, 1 H, H-9, Fmoc), 4.52 (d, ^3J = 6.3 Hz, 2 H, 9-CH_2, Fmoc), 7.24–7.40 (m, 4 H, H_{arom}, Fmoc), 7.60–7.90 (m, 7 H, H_{arom}, Fmoc).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 46.5, 66.1, 112.0, 119.3, 120.2, 120.5, 125.0, 127.1, 127.7, 131.4, 140.8, 142.9, 143.5, 149.9, 153.2, 165.0.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{22}H_{15}N_2O_8$ + 2 Na: 449.0720; found: 449.0730.

Di-*tert*-butyl *N*-(4-{[(9*H*-Fluorene-9-yl)methoxy]carbonylamino}-2-nitrobenzoyl)-L-glutamate (10)

HBTU (17.7 g, 46.7 mmol) was added to a mixture of **9** (17.2 g, 42.5 mmol) and Et_3N (12.9 mL) in CH_2Cl_2 (222 mL). After stirring for 15 min at r.t., a mixture of di-*tert*-butyl L-glutamate hydrochloride (13.8 g, 46.7 mmol) in CH_2Cl_2 (172 mL) was added. The reaction mixture was stirred under N_2 at r.t. for 20 h. After the addition of MTBE (860 mL), the mixture was washed with 5% aq $NaHCO_3$ (5 × 170 mL), 5% aq citric acid (5 × 170 mL), and brine (2 × 425 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness under vacuum to give crude **10** (27.9 g). Purification was accomplished by chromatography on silica gel (EtOAc–*n*-heptane, 45:55) to give **10**; yield: 23.8 g (87%); light yellow foam; R_f = 0.68 (EtOAc–*n*-heptane, 7:3).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.40 (s, 9 H, *t*-C_4H_9), 1.43 (s, 9 H, *t*-C_4H_9), 1.72–2.09 [m, 2 H, C(β)H_2, Glu], 2.34 [t, ^3J = 7.6 Hz, 2 H, C(γ)H_2, Glu], 4.22–4.37 [m, 2 H, C(α)H, Glu, H-9, Fmoc], 4.58 (d, ^3J = 6.4 Hz, 2 H, 9-CH_2, Fmoc), 7.31–7.54 (m, 5 H, H_{arom}, Fmoc), 7.74–8.14 (m, 11 H, H_{arom}, Fmoc), 8.86 (br d, 1 H, NH, Glu), 10.3 (br s, 1 H, NH, Fmoc).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.0, 27.5, 27.7, 30.9, 46.5, 52.2, 66.0, 79.7, 80.7, 112.7, 120.1, 121.5, 125.0, 125.3, 127.1, 127.0, 127.6, 140.7, 141.1, 143.5, 147.7, 153.2, 165.0, 170.4, 171.4.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{35}H_{39}N_3O_9$ + Na: 668.2579; found: 668.2599.

Di-*tert*-butyl *N*-(4-Amino-2-nitrobenzoyl)-L-glutamate (11)

Pyrrolidine (3.7 mL) was added to **10** (25 g, 38.7 mmol) in DMF (500 mL). After 30 min at r.t., the solution was evaporated to dryness at 40 °C under vacuum to give a yellow tar (25.0 g). After complete removal of residual DMF under high vacuum, $(i\text{-Pr})_2O$ (500 mL) was added. After cooling to 0 °C and stirring for 3 h, the resulting solid was drawn off by suction, washed with $(i\text{-Pr})_2O$ (150 mL), and dried under vacuum at 40 °C to give **11**, which was used without further purification for the synthesis of **13**; yield: 12.6 g (77%); light yellow crystals; mp 120 °C.

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.40 (s, 9 H, *t*-C_4H_9), 1.41 (s, 9 H, *t*-C_4H_9), 1.71–1.96 [m, 2 H, C(β)H_2, Glu], 2.32 [t, ^3J = 7.5 Hz, 2 H, C(γ)H_2, Glu], 4.1–4.26 [m, 1 H, C(α)H, Glu], 6.12 (br s, 2 H, NH_2), 6.78 (dd, ^3J = 8.4 Hz, ^4J = 2.3 Hz, 1 H, 5′-H_{arom}), 6.97 (d, ^4J = 2.2 Hz, 1 H, 3′-H_{arom}), 7.32 (d, ^3J = 8.4 Hz, 1 H, 6′-H_{arom}), 8.59 (br d, ^3J = 7.7 Hz, 1 H, NH, Glu).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.0, 27.6, 27.7, 31.1, 52.2, 79.7, 80.6, 107.5, 115.5, 117.0, 130.1, 149.8, 151.2, 165.3, 170.7, 171.5.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{20}H_{29}N_3O_7$ + Na: 446.1898; found: 446.1900.

N^2-(*N*,*N*-Dimethylaminomethylene)-2′-nitrofolic Acid Di-*tert*-butyl Ester (13)

6-(Bromomethyl)pterin hydrobromide[24,25] (**12**; 26.5 g, 78.6 mmol) was added to a solution of **11** (12.6 g, 29.8 mmol) in DMAC (130 mL). The mixture was stirred under N_2 at 60 °C for 13 h. After cooling to r.t., the mixture was filtered and the filtrate was added dropwise to H_2O (8800 mL). The crystals were drawn off by suction, washed with H_2O (880 mL), and dried at 35 °C under vacuum to give crude 2′-nitrofolic acid di-*tert*-butyl ester (14.1 g). *N*,*N*-Dimethylformamide diisopropylacetal (31 mL) was added to a solution of crude 2′-nitrofolic acid di-*tert*-butyl ester (8.8 g) in anhyd DMF (133 mL). The mixture was stirred under N_2 for 20 h at r.t. and then evaporated to dryness. The residue was purified three times by chromatography on silica gel (CH_2Cl_2–MeOH, 95:5) to give **13**; yield: 2.75 g (14% corresponding to **11**); orange-yellow solid; R_f = 0.58 (CH_2Cl_2–MeOH, 85:15).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.39 (s, 9 H, *t*-C_4H_9), 1.40 (s, 9 H, *t*-C_4H_9), 1.70–2.05 [m, 2 H, C(β)H_2, Glu], 2.31 [t, ^3J = 7.4 Hz, 2 H, C(γ)H_2, Glu], 3.09 (s, 3 H, NCH_3), 3.22 (s, 3 H, NCH_3) 4.14–4.26 [m, 1 H, C(α)H, Glu], 4.59 (d, ^3J = 5.9 Hz, 2 H, H-6), 6.91 (dd, ^3J = 8.5 Hz, ^4J = 2.3 Hz, 1 H, 5′-H_{arom}), 7.12 (d, ^3J = 2.2 Hz, 1 H, 6′-H_{arom}), 7.37 (d, ^3J = 8.3 Hz, 1 H, 3′-H_{arom}), 7.42 (br s, 1 H, NH), 8.61 (br d, ^3J = 7.7 Hz, 1 H, NH), 8.75, 8.79 (2 s, 2 H, H-7, CH=N), 12.00 [br s, 1 H, N(3)H].

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.0, 27.6, 27.7, 31.0, 34.9, 40.9, 45.8, 52.2, 79.7, 80.6, 106.7, 114.1, 117.6, 129.9, 130.2, 148.6, 149.0, 149.8, 150.1, 155.5, 159.1, 165.2, 170.7, 171.4.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{30}H_{39}N_9O_8$ + Na: 676.2814; found: 676.2816.

4-(*tert*-Butoxycarbonylamino)-2-nitrobenzoic Acid (15)

An aqueous solution of NaOH (32%, 0.69 g) was added to a suspension of 4-amino-2-nitrobenzoic acid[26] (**8**; 1 g, 5.5 mmol) in H_2O (10 mL), followed by the addition of a solution of di-*tert*-butyl dicarbonate (1.2 g, 5.5 mmol) in 1,4-dioxane (12 mL). After 29 h at r.t., additional di-*tert*-butyl dicarbonate (0.24 g, 1.1 mmol) was added, and the mixture was stirred for a further 2 h at r.t. The reaction mixture was washed with MTBE (3 × 5 mL). The pH of the aqueous layer was adjusted to 3 by the addition of 10% aq citric acid (14.8 g) and the resulting suspension was cooled to 0 °C. The product was drawn off by suction, washed with H_2O (50 mL), and dried at 40 °C under vacuum to give **15**; yield: 0.72 g (47%); off-white crystals; >180 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.46 (s, 9 H, *t*-C_4H_9), 7.64 (dd, ^3J = 8.6 Hz, ^4J = 2.0 Hz, 1 H, 5′-H_{arom}), 7.79 (d, ^3J = 8.5 Hz, 1 H, 6′-H_{arom}), 7.97 (d, ^4J = 1.9 Hz, 1 H, 3′-H_{arom}), 10.08 (br s, 1 H, NH).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 27.9, 80.5, 111.6, 118.8, 120.2, 131.3, 143.4, 150.0, 152.4, 165.0.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{12}H_{13}N_2O_6$ + 2 Na: 327.0564; found: 327.0574.

4-(*tert*-Butoxycarbonylamino)-2-fluorobenzoic Acid (16)

An aqueous solution of NaOH (32%, 6.4 g) was added to a suspension of 4-amino-2-fluorobenzoic acid[30] (**14**; 8.1 g, 52.2 mmol) in H_2O (80 mL), followed by the addition of a solution of di-*tert*-butyl dicarbonate (28.3 g, 129.7 mmol) in 1,4-dioxane (80 mL). After 48 h at r.t., the reaction mixture was washed with MTBE (5 × 50 mL). The pH of the aqueous layer was adjusted to 5.8 by the addition of 10% aq citric acid. The resulting suspension was cooled to 0 °C and stirred for 1 h. The product was drawn off by suction, washed with H_2O (120 mL) and dried at 40 °C under vacuum to give **16**; yield: 7.3 g (55%); colorless crystals; mp >200 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.50 (s, 9 H, *t*-C_4H_9), 7.29–7.49 (m, 2 H, 5′-H_{arom}, 6′-H_{arom}), 7.80 (m, 1 H, 3′-H_{arom}), 9.92 (br s, 1 H, NH), 12.83 (br s, 1 H, CO_2H).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 27.9, 39.5, 80.1, 104.8, 105.3, 111.8, 112.0, 113.2, 132.7, 145.3, 145.5, 152.3, 159.4, 164.5, 164.6, 164.7.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{12}H_{14}FNO_4$ + Na: 278.0799; found: 278.0794.

Di-*tert*-butyl *N*-[4-(*tert*-Butoxycarbonylamino)-2-nitrobenzoyl]-L-glutamate (17)

A solution of di-*tert*-butyl L-glutamate hydrochloride (3.8 g, 12.8 mmol) and Et_3N (1.5 mL) in CH_2Cl_2 (60 mL) was added to a mixture of **15** (3 g, 10.6 mmol), Et_3N (1.5 mL), HBTU (4.8 g, 12.7 mmol), and CH_2Cl_2 (60 mL). The mixture was stirred at r.t. for 18 h. Solids were removed by filtration and the filtrate was washed with 5% aq citric acid (3 × 50 mL), sat. aq $NaHCO_3$ (3 × 50 mL), and H_2O (2 × 50 mL). The organic layer was dried ($MgSO_4$) and

evaporated to dryness at 40 °C under vacuum to give crude **17** (6.3 g) as a yellow foam. Purification was accomplished by chromatography on silica gel (EtOAc–*n*-heptane, 4:6); yield: 5.1 g (92%); bright yellow foam; R_f = 0.44 (EtOAc–MeOH, 90:10).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.40 (s, 9 H, *t*-C_4H_9, Glu), 1.43 (s, 9 H, *t*-C_4H_9, Glu), 1.50 (s, 9 H, *t*-C_4H_9, Boc), 1.72–2.08 [m, 2 H, C(β)H_2, Glu], 2.34 [t, ^3J = 7.6 Hz, 2 H, C(γ)H_2, Glu], 4.22–4.33 [m, 1 H, C(α)H, Glu], 7.51 (d, ^3J = 8.4 Hz, 1 H, 6′-H_{arom}), 7.72 (dd, d, ^4J = 2.1 Hz, ^3J = 8.4 Hz, 1 H, 5′-H_{arom}), 8.16 (d, ^4J = 2.0 Hz, 1 H, 3′-H_{arom}), 8.84 (d, ^2J = 7.7 Hz, NH, Glu), 10.01 (br s, 1 H, NH).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.1, 27.6, 27.7, 27.9, 31.0, 52.2, 79.7, 80.2, 80.7, 112.4, 121.3, 124.8, 129.7, 141.7, 147.8, 152.5, 165.1, 170.4, 171.4.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{25}H_{37}N_3O_9$ + Na: 546.2422; found: 546.2444.

Di-*tert*-butyl *N*-[4-(*tert*-Butoxycarbonylamino)-2-fluorobenzoyl]-L-glutamate (18)

A solution of di-*tert*-butyl L-glutamate hydrochloride (4.17 g, 14.1 mmol) and Et_3N (1.64 mL) in CH_2Cl_2 (120 mL) was added to a mixture of **16** (3 g, 11.8 mmol), Et_3N (1.64 mL), HBTU (5.35 g, 14.1 mmol), and CH_2Cl_2 (120 mL). The mixture was stirred at r.t. for 18 h. Solids were removed by filtration and the filtrate was washed with 5% aq citric acid (5 × 60 mL), sat. aq $NaHCO_3$ (3 × 60 mL) and H_2O (2 × 60 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness at 40 °C under vacuum to give 7.03 g crude **18** (7.03 g) as a yellow foam. Purification was accomplished by chromatography on silica gel (EtOAc–*n*-heptane, 3:7); yield: 5.3 g (91%); colorless crystals; mp 117 °C; R_f = 0.50 (EtOAc–MeOH, 1:1).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.39 (s, 9 H, *t*-C_4H_9, Glu), 1.42 (s, 9 H, *t*-C_4H_9, Glu), 1.49 (s, 9 H, *t*-C_4H_9, Boc), 1.78–2.11 [m, 2 H, C(β)H_2, Glu], 2.28–2.36 [m, 2 H, C(γ)H_2, Glu], 4.26–4.37 [m, 1 H, C(α)H, Glu], 7.28 (dd, $^3J_{H,F}$ = 8.6 Hz, ^4J = 2.0 Hz, 1 H, 3′-H_{arom}), 7.44 (dd, ^4J = 1.9 Hz, ^3J = 13.7 Hz, 1 H, 5′-H_{arom}), 7.55 (m, 1 H, 6′-H_{arom}), 8.20 (dd, ^4J = 3.4 Hz, ^3J = 7.5 Hz, 1 H, $C_α$-NH), 9.80 (br s, 1 H, C4′-NH).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.1, 27.6, 27.7, 27.9, 31.0, 52.2, 79.7, 80.2, 80.7, 112.4, 121.2, 124.8, 129.7, 141.6, 147.8, 152.5, 165.1, 170.4, 171.4.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{25}H_{37}FN_2O_7$ + Na: 519.2477; found: 519.2480.

Dimethyl *N*-[4-(*tert*-Butoxycarbonylamino)-2-nitrobenzoyl]-L-glutamate (19)

A slurry of dimethyl L-glutamate hydrochloride (13.3 g, 62.8 mmol) and Et_3N (7.3 mL) in CH_2Cl_2 (296 mL) was added to a mixture of **15** (14.8 g, 52.4 mmol), Et_3N (7.3 mL), HBTU (23.9 g, 63.0 mmol), and CH_2Cl_2 (296 mL). The mixture was stirred at r.t. for 18 h. Solids were removed by filtration and the filtrate was washed times with 5% aq citric acid (5 × 100 mL), sat. aq $NaHCO_3$ (5 × 100 mL), and H_2O (100 mL). The organic layer was dried ($MgSO_4$) and evaporated to dryness at 40 °C under vacuum to give crude **19** (27.4 g) as an orange tar. Purification was accomplished by chromatography on silica gel (EtOAc–*n*-heptane, 35:65) to give pure **19**; yield: 17.4 g (76%); bright yellow foam; R_f = 0.28 (EtOAc–*n*-heptane, 7:3).

^{1}H NMR (200 MHz, $CDCl_3$): δ = 1.53 (s, 9 H, *t*-C_4H_9), 2.07–2.45 [2 m, 2 H, C(β)H_2, Glu], 2.55 [m, 2 H, C(γ)H_2, Glu], 3.68 (s, 3 H, OCH_3), 3.80 (s, 3 H, OCH_3), 4.76–4.86 [m, 1 H, C(α)H, Glu], 6.71 (d, ^3J = 7.7 Hz, 1 H, $C_α$-NH) 6.95 (br s, 1 H, NH), 7.45 (d, ^3J = 8.4 Hz, 1 H, 6′-H_{arom}), 7.62 (dd, ^4J = 2.2 Hz, ^3J = 8.4 Hz, 5′-H_{arom}), 8.12 (d, ^4J = 2.1 Hz, 1 H, 3′-H_{arom}).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 27.0, 28.2, 30.0, 51.9, 52.3, 52.7, 81.8, 113.8, 122.2, 125.4, 129.3, 141.3, 147.3, 152.3, 166.3, 172.0, 173.6.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{25}N_3O_9$ + Na: 462.1483; found: 462.1478.

N-(4-Amino-2-nitrobenzoyl)-L-glutamic Acid (20)

Trifluoroacetic acid (50 mL) was added to a solution of **17** (5.3 g, 10.1 mmol) in anhyd CH_2Cl_2 (50 mL) at 0 °C. After removal of the cooling bath, the mixture was stirred at r.t. and then evaporated to dryness to give **20** as the trifluoroacetate. The product was not further purified; yield: 3.4 g (79%); orange tar.

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.77–2.14 [m, 2 H, C(β)H_2, Glu], 2.36 [t, ^3J = 7.5 Hz, 2 H, C(γ)H_2, Glu], 4.26–4.38 [m, 1 H, C(α)H, Glu], 6.80 (dd, ^3J = 8.4 Hz, ^4J = 2.3 Hz, 1 H, 5′-H_{arom}), 6.99 (d, ^4J = 2.2 Hz, 1 H, 3′-H_{arom}), 7.36 (d, ^3J = 8.4 Hz, 1 H, 6′-H_{arom}), 8.57 (d, ^3J = 7.9 Hz, 1 H, $C_α$-NH), 9.68 (br s, 3 H, NH_3^+ and H_2O present in DMSO-d_6).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.0, 27.6, 27.7, 31.1, 52.2, 79.7, 80.6, 107.5, 115.5, 117.0, 130.1, 149.8, 151.2, 165.3, 170.7, 171.5.

HRMS (ESI, CH_2Cl_2): *m*/*z* [M + H]$^+$ calcd for $C_{12}H_{14}N_3O_7$: 312.0826; found: 312.0826.

N-(4-Amino-2-fluorobenzoyl)-L-glutamic Acid (21)

Trifluoroacetic acid (50 mL) was added to a solution of **18** (5.2 g, 10.5 mmol) in anhyd CH_2Cl_2 (50 mL) at 0 °C. After removal of the cooling bath, the mixture was stirred at r.t., and then evaporated to dryness to give **21** as the trifluoroacetate. The product was not further purified; yield: 4.0 g (96%); light brown foam.

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.83–2.17 [m, 2 H, C(β)H_2, Glu], 2.28–2.36 [m, 2 H, C(γ)H_2, Glu], 4.35–4.45 [m, 1 H, C(α)H, Glu], 6.35 (dd, ^4J = 2.0 Hz, ^3J = 14.3 Hz, 1 H, 3′-H_{arom}), 6.45 (dd, ^4J = 2.1 Hz, ^3J = 8.5 Hz, 1 H, 5′-H_{arom}), 7.47 (t, ^3J = 8.7 Hz, 1 H, 6′-H_{arom}), 7.71 (m, 1 H, $C_α$-NH), 10.66 (br s, 3 H, NH_3^+ and H_2O present in DMSO-d_6).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.2, 30.1, 51.7, 99.4, 99.9, 108.6, 109.8, 131.8, 152.9, 153.1, 163.5, 173.3, 173.8.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{12}H_{12}FN_2O_5$ + 2 Na: 329.0520; found: 329.0519.

Dimethyl *N*-(4-Amino-2-nitrobenzoyl)-L-glutamate (22)

Trifluoroacetic acid (30 mL) was added to a solution of **19** (17.0 g, 38.7 mmol) in anhyd CH_2Cl_2 (170 mL) at 0 °C. After removal of the cooling bath, the mixture was stirred at r.t. for 5 h, and then evaporated to dryness to give crude **22** (19.5 g) as a yellow highly viscous oil. Purification was accomplished by two-fold chromatography on silica gel (CH_2Cl_2–MeOH, 95:5) to give a bright yellow foam (8.6 g), which was treated with (*i*-Pr)$_2$O (172 mL). After stirring for 2 h, the crystals were drawn off by suction, washed with (*i*-Pr)$_2$O (33 mL), and dried at 35 °C overnight under vacuum to give pure **22** as the trifluoroacetate; yield: 7.9 g (45%); yellow crystals; mp 126 °C; R_f = 0.46 (CH_2Cl_2–MeOH, 90:10).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.82–2.18 [m, 2 H, C(β)H_2, Glu], 2.45 [t, ^3J = 7.7 Hz + DMSO-signal, 2 H, C(γ)H_2, Glu], 3.60 (s, 3 H, OCH_3), 3.65 (s, 3 H, OCH_3), 4.32–4.43 [m, 1 H, C(α)H, Glu], 6.07 (br s, H_2O and/or NH_2), 6.79 (dd, ^3J = 8.4 Hz, ^4J = 2.1 Hz, 1 H, 5′-H_{arom}), 6.99 (d, ^4J = 2.1 Hz, 1 H, 3′-H_{arom}), 7.34 (d, ^3J = 8.4 Hz, 1 H, 6′-H_{arom}), 8.71 (d, ^3J = 7.6 Hz, 1 H, $C_α$-NH).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 25.9, 29.7, 51.3, 51.5, 51.9, 107.5, 115.6, 116.7, 130.1, 149.8, 151.3, 165.5, 171.9, 172.6.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{14}H_{17}N_3O_7$ + Na: 362.0959; found: 362.0961.

2′-Nitrofolic Acid (2)

6-(Bromomethyl)pterin hydrobromide[24,25] (**12**; 2.3 g, 6.8 mmol) was added to a solution of crude **20** (3.4 g, 7.99 mmol) in anhyd DMAC (50 mL). The mixture was stirred for 5 h at 60 °C and for 17 h at r.t. The solids were removed by filtration and the filtrate was added to aq 0.1 M HCl (321 mL). The resulting suspension was stirred for 1 h at r.t., the product was drawn off by suction, washed with aq 0.1 M HCl (24 mL) and H_2O (24 mL), and dried under vacuum at 40 °C to give crude **2** (1.54 g). The crude product was dissolved in H_2O (26 mL) at 70 °C by the addition of 37% aq HCl (15.4 mL). The solution was treated with charcoal (0.31 g) at 70 °C and after removal of charcoal, the filtrate was cooled to r.t. After the addition of an aq solution of NaOH (32%, 16.6 g), the mixture was stirred for 30 min at 0 °C. The product was drawn off by suction, washed with H_2O (20 mL), and dried at 40 °C under vacuum to give pure **2**; yield: 0.86 g (26% corresponding to **12**); greenish-yellow crystals; mp >200 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.71–2.09 [2 m, 2 H, C(β)H_2, Glu], 2.31 [t, ^3J = 7.4 Hz, 2 H, C(γ)H_2, Glu], 4.21–4.32 [m, 1 H, C(α)H, Glu], 4.54 (s, 2 H, H-6), 6.86 (dd, ^3J = 8.6 Hz, ^4J = 2.3 Hz, 1 H, 5′-H_{arom}), 7.07 (d, ^4J = 2.3 Hz, 1 H, 3′-H_{arom}), 7.21 (br s, 1 H, NH_2), 7.37 (d, ^3J = 8.5 Hz, 1 H, 6′-H_{arom}), 8.60 (d, ^3J = 7.8 Hz, 1 H, $C_α$-NH), 8.68 (s, 1 H, H-7).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.2, 30.2, 45.7, 51.6, 106.7, 114.2, 117.6, 128.1, 130.2, 148.5, 149.9, 150.1, 153.6, 160.5, 165.2, 173.1, 173.8.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{17}N_8O_8$ 2 Na: 531.0959; found: 531.0955.

2′-Fluorofolic Acid (3)

6-(Bromomethyl)pterin hydrobromide[24,25] (**12**; 1.7 g, 5.0 mmol) was added to a solution of crude **21** (2 g, 5.0 mmol) in anhyd DMAC (76 mL). The mixture was stirred for 6 h at 60 °C. After cooling to r.t., solids were removed by filtration. The filtrate was added to 0.1 M aq HCl (486 mL). After cooling to 4 °C for 17 h, the crystallized product was drawn off by suction, washed with 0.1 M aq HCl (29 mL) and H_2O (90 mL), and dried under vacuum at 40 °C to give 0.76 g of crude **3**, which was dissolved in H_2O (11 mL) at r.t. by the addition of 37% aq HCl (7.6 mL). After cooling to 4 °C for 5 h, the crystallized product was drawn off by suction, washed with aq 0.05 M HCl (20 mL) and H_2O (20 mL), and dried under vacuum at 40 °C to give pure **3**, yield: 0.68 g (30% corresponding to **12**); yellowish crystals; mp >230 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.82–2.09 [m, 2 H, C(β)H_2, Glu], 2.28 [t, ^3J = 7.4 Hz, 2 H, C(γ)H_2, Glu], 4.26–4.37 [m, 1 H, C(α)H, Glu], 4.49 (d, ^3J = 5.7 Hz, 2 H, H-6), 6.39–6.55 (2 dd, 2 H, 3′-$H_{arom,}$ 5′-H_{arom}), 7.03 (br s, 1 H, NH_2), 7.20 [t, ^3J = 5.7 Hz, 1 H, C(6)-NH], 7.51 (m, 1 H, 6′-H_{arom}), 7.75 (t, ^3J = 7.2 Hz, 1 H, $C_α$-NH), 8.65 (s, 1 H, H-7), 11.66 [br s, 1 H, N(3)-H].

^{13}C NMR (100 MHz, DMSO-d_6): δ = 26.2, 30.1, 45.8, 51.7, 97.9 ($^2J_{C,F}$ = 28 Hz), 108.4, 108.9 ($^2J_{C,F}$ = 12 Hz), 127.9, 131.6, 147.9, 148.5, 152.5 ($^3J_{C,F}$ = 12 Hz), 153.8, 156.4 (br), 160.9 (br), 161.6 ($^1J_{C,F}$ = 246 Hz), 163.3, 173.2, 173.7.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{19}H_{17}FN_7O_6$: 458.1230; found: 458.1231.

2′-Nitrofolic Acid Dimethyl Ester (23)

Method A: 6-(Bromomethyl)pterin hydrobromide[24,25] (**12**; 5.5 g, 16.3 mmol) was added to a solution of **22** (5.0 g, 11.0 mmol) in DMAC (500 mL). The mixture was stirred for 9 h at 60 °C. After cooling to r.t., the solids were removed by filtration, and the filtrate was added dropwise to 0.1 M aq HCl (3500 mL) at r.t. After cooling to 0 °C overnight, the crystallized product was drawn off by suction, washed with 0.1 M aq HCl (50 mL) and H_2O (300 mL), and dried under vacuum at 40 °C to give **23** (2.1 g). A second crop of **23** (0.8 g) was obtained from the mother liquor; total yield: 2.9 g (49%); citreous-greenish crystals; mp >250 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.80–2.15 [m, 2 H, C(β)H_2, Glu], 2.44 [t, ^3J = 7.5 Hz + DMSO-signal, 2 H, C(γ)H_2, Glu], 3.60 (s, 3 H, OCH_3), 3.65 (s, 3 H, OCH_3), 4.32–4.42 [m, 1 H, C(α)H, Glu], 4.56 (d, ^3J = 5.9 Hz, 2 H, H-6), 6.90 (dd, d, ^4J = 2.2 Hz, ^3J = 8.6 Hz, 1 H, 5′-H_{arom}), 7.05 (br s, 2 H, NH_2), 7.11 (d, ^4J = 2.2 Hz, 1 H, 3′-H_{arom}), 7.39 (d + NH-signal, ^3J = 8.5 Hz, 2 H, 6′-H_{arom}), 8.68 (s, 1 H, H-7), 8.74 (d, ^3J = 7.8 Hz, 1 H, NH, Glu), 11.50 [br s, 1 H, N(3)H].

^{13}C NMR (50 MHz, DMSO-d_6): δ = 25.9, 29.7, 45.7, 51.3, 51.6, 51.9, 106.7, 114.2, 117.3, 128.1, 130.2, 147.7, 148.5, 149.8, 150.2, 153.8, 155.7, 160.8, 165.4, 171.9, 172.6.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{21}H_{22}N_8O_8$ + Na: 537.1453; found: 537.1454.

Method B: 2′-Nitrofolic acid (**2**; 200 mg, 0.4 mmol) was added to a solution of benzenesulfonic acid (130 mg) in MeOH (5 mL). The yellow slurry was stirred at 90 °C for 2 h and cooled to 0 °C overnight. The crystallized product was drawn off by suction, washed with ice-cold MeOH (2 mL), and dried overnight at 40 °C under vacuum to give **23** as hemibenzenesulfonate; yield: 175 mg (72%); greenish-yellow crystals; mp >250 °C (dec.); BSA content (determined with HPLC using a reference standard): 11% w/w corresponding to approx. 0.5 mol BSA/1 mol 2′-nitrofolic acid dimethyl ester.

^{1}H NMR (200 MHz, DMSO-d_6 + 3 drops of D_2O): δ = 1.81–2.16 [m, 2 H, C(β)H_2, Glu], 2.44 [m + DMSO-signal, 2 H + DMSO-signal, C(γ)H_2, Glu], 3.60 (s, 3 H, OCH_3), 3.65 (s, 3 H, OCH_3), 4.37 [m, 1 H, C(α)H, Glu], 4.61 (s, 2 H, H-6), 6.92 (dd, ^4J = 1.8 Hz, ^3J = 8.4 Hz, 1 H, 5′-H_{arom}), 7.12 (d, ^4J = 1.7 Hz, 1 H, 3′-H_{arom}), 7.38 (d, m, 1 H, 6′-$H_{arom,}$ +H_{arom} from $PhSO_3H$), 7.64 (dd, ^3J = 6.5 Hz, ^4J = 2.8 Hz, 1 H, NH), 8.75 (s, 1 H, H-7).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 25.8, 29.6, 45.5, 51.3, 51.5, 51.9, 106.7, 114.2, 117.4, 125.4, 127.7, 128.1, 128.6, 130.2, 148.1, 149.6, 149.8, 150.1, 152.0, 152.9, 159.8, 165.3, 171.8, 172.6.

HRMS (ESI, MeOH–H_2O, 1:1): *m*/*z* [M + H]$^+$ calcd for $C_{21}H_{22}N_8O_8$ + Na: 537.1453; found: 537.1439.

N^{10}-Formyl-2′-nitrofolic Acid Dimethyl Ester (24)

2′-Nitrofolic acid dimethyl ester (**23**; 1.9 g, 3.69 mmol) was stirred in formic acid (98%, 190 mL) at 60 °C for 3.5 h. The solution was concentrated to a volume of 20 mL and then ice-cold H_2O (100 mL) was added dropwise. The crystallized product was drawn off by suction, washed with ice-cold H_2O (4 mL) and dried at 40 °C under vacuum for 17 h to give **24**; yield: 1.54 g (77%); green crystals; mp >250 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.81– 2.17 [m, 2 H, C(β)H_2, Glu], 2.42 [m, 2 H + DMSO-signal, C(γ)H_2, Glu], 3.60 (s, 3 H, OCH_3), 3.67 (s, 3 H, OCH_3), 4.45 [m, 1 H, C(α)H, Glu], 5.24 (s, 2 H, H-6), 6.89 (br s, 2 H, NH_2), 7.62 (d, ^3J = 8.3 Hz, 1 H, 6′-H_{arom}), 7.90 (dd, ^4J = 2.0 Hz, ^3J = 8.4 Hz, 1 H, 5′-H_{arom}), 8.22 (d, ^4J = 2.0 Hz, 1 H, 3′-H_{arom}), 8.66 (s, 1 H, H-7), 8.84 (s, 1 H, CHO), 9.10 (d, ^3J = 7.7 Hz, 1 H, NH, Glu), 11.43 [br s, 1 H, N(3)H].

^{13}C NMR (100 MHz, DMSO-d_6): δ = 25.8, 29.5, 46.4, 51.3, 51.5, 52.0, 117.3, 126.0, 128.0, 130.1, 142.9, 145.0, 148.0, 148.8, 153.8, 156.5, 160.5, 162.5, 164.8, 171.4, 172.5.

HRMS (ESI, MeOH–H_2O, 1:1): *m*/*z* [M + H]$^+$ calcd for $C_{22}H_{23}N_8O_9$: 543.1583; found: 543.1584.

N^2-(*N*,*N*-Dimethylaminomethylene)-N^{10}-formyl-2′-nitrofolic Acid Dimethyl Ester (25)

N,*N*-Dimethylformamide diisopropylacetal (5.4 mL) was added dropwise to **24** (1.34 g) in anhyd DMF (45 mL) within 30 min at r.t.

The mixture was stirred at r.t. for 17 h and evaporated to dryness. The residue (1.75 g) was purified by column chromatography on silica gel (CH_2Cl_2–MeOH, 93:7) to give **25**; yield: 0.97 g (66%); off-white crystals; mp >180 °C (dec.); R_f = 0.4 (CH_2Cl_2–MeOH, 9:1).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.83–2.19 [m, 2 H, C(β)H_2, Glu], 2.47 [m, 2 H + DMSO-signal, C(γ)H_2, Glu], 3.09 (s, 3 H, NCH_3), 3.22 (s, 3 H, NCH_3), 3.61 (s, 3 H, OCH_3), 3.68 (s, 3 H, OCH_3), 4.47 [m, 1 H, C(α)H, Glu], 5.30 (s, 2 H, H-6), 7.65 (d, ^3J = 8.3 Hz, 1 H, 6′-H_{arom}), 7.93 (dd, ^4J = 2.0 Hz, ^3J = 8.4 Hz, 1 H, 5′-H_{arom}), 8.25 (d, ^4J = 1.9 Hz, 1 H, 3′-H_{arom}), 8.77 (s, 1 H, H-7), 8.78 (s, 1 H, CH=N), 8.88 (s, 1 H, CHO), 9.12 (d, ^3J = 7.7 Hz, 1 H, NH, Glu), 11.99 [br s, 1 H, N(3)H].

^{13}C NMR (100 MHz, DMSO-d_6): δ = 25.8, 29.5, 34.9, 40.9, 46.7, 51.2, 51.5, 51.9, 117.2, 125.8, 128.0, 130.1, 142.8, 146.4, 147.9, 148.7, 155.4, 158.1, 159.1, 161.3, 162.5, 164.7, 171.4, 172.4.

HRMS (ESI, CH_2Cl_2–MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{25}H_{28}N_9O_9$: 598.2004; found: 598.2003.

2′-Fluorofolic Acid Dimethyl Ester Benzenesulfonate (26)

2′-Fluorofolic acid (**3**; 2.92 g, 6.36 mmol) was added to a solution of benzenesulfonic acid (2.1 g, 13.3 mmol) in MeOH (73 mL). The mixture was heated to reflux for 2 h and then kept at 5 °C for 17 h. The crystallized product was drawn off by suction, washed with MeOH (15 mL), and dried under vacuum at 40 °C to give **26**; yield: 3.12 g (76%); greenish-yellow crystals; mp >80 °C (dec.); BSA content (measured by HPLC against a standard): 23% w/w corresponding to approx. 1 mol BSA/1 mol 2′-fluorofolic acid dimethyl ester.

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.86–2.18 [m, 2 H, C(β)H_2, Glu], 2.40 [t, ^3J = 7.4 Hz, 2 H, C(γ)H_2, Glu], 3.57 (s, 3 H, OCH_3), 3.63 (s, 3 H, OCH_3), 4.42 [m, 1 H, C(α)H, Glu], 4.58 (s, 2 H, H-6), 6.44 (dd, ^4J = 2.0 Hz, ^3J = 14.4 Hz, 1 H, 3′-H_{arom}), 6.53 (dd, ^4J = 2.1 Hz, ^3J = 8.6 Hz, 1 H, 5′-H_{arom}), 7.34 (m, H_{arom}, $PhSO_3H$), 7.46 (t, ^3J = 8.7 Hz, 1 H, 6′-H_{arom}), 7.63 (m, H_{arom}, $PhSO_3H$), 7.95 (dd, br s, ^3J = 7.2 Hz, ^4J = 5.2 Hz, 3 H, NH_2, NH, Glu), 8.76 (s, 1 H, H-7).

^{13}C NMR (50 MHz, DMSO-d_6): δ = 25.8, 29.7, 45.5, 51.3, 51.7, 51.9, 97.9, 98.4, 108.5, 109.1, 109.3, 125.4, 127.8, 128.0, 128.9, 131.6 (d), 147.1, 147.7, 147.9, 151.7, 152.2, 158.7, 159.0, 163.7, 163.9, 172.1, 172.6.

HRMS (ESI, MeOH): *m*/*z* [M + H]$^+$ calcd for $C_{21}H_{23}FN_7O_6$: 488.1688; found: 488.1690.

2′-Fluoro-N^{10}-formylfolic Acid Dimethyl Ester (27)

Benzenesulfonate **26** (2.5 g, 3.87 mmol) was stirred in formic acid (98%, 250 mL) for 4 h at 60 °C. The reaction mixture was concentrated to 50 mL under vacuum at 40 °C and treated with ice-cold H_2O (230 mL). The crystallized product was drawn off by suction, washed with ice-cold H_2O (50 mL), and dried under vacuum at 40 °C to give **27**; yield: 1.7 g (85%); brownish crystals; mp >250 °C (dec.).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.87–2.20 [m, 2 H, C(β)H_2, Glu], 2.46 [t, ^3J = 7.6 Hz, 2 H + DMSO-signal, C(γ)H_2, Glu], 3.60 (s, 3 H, OCH_3), 3.67 (s, 3 H, OCH_3), 4.48 [m, 1 H, C(α)H, Glu], 5.23 (s, 2 H, H-6), 6.98 (br s, 2 H, NH_2), 7.42 (d, *J* = 7.9 Hz, 1 H, 3′-H_{arom}), 7.62 (m, 2 H, 6′-H_{arom}, NH, Glu), 8.65 (m, 2 H, 5′-H_{arom}, CHO), 8.85 (s, 1 H, H-7), 11.54 [br s, 1 H, N(3)H].

^{13}C NMR (50 MHz, DMSO-d_6): δ = 26.4, 30.4, 47.1, 52.0, 52.5, 52.7, 105.0, 110.1, 110.6, 118.1 (d), 120.7, 121.0, 128.8, 131.7, 145.0, 145.2, 146.1, 149.4, 154.6, 157.8, 162.8, 164.1, 172.5, 173.3.

HRMS (ESI, MeOH–H_2O, 1:1): *m*/*z* [M + H]$^+$ calcd for $C_{22}H_{23}FN_7O_7$: 516.1638; found: 516.1654.

N^2-(*N*,*N*-Dimethylaminomethylene)-2′-fluoro-N^{10}-formylfolic Acid Dimethyl Ester (28)

N,*N*-Dimethylformamide diisopropylacetal (6.1 mL) was added dropwise to **27** (1.5 g, 2.9 mmol) in anhyd DMF (50 mL) within 30 min at r.t. The mixture was stirred at r.t. for 17 h and evaporated to dryness. The residue (2.08 g) was purified by column chromatography on silica gel (CH_2Cl_2–MeOH, 93:7) to give **28**; yield: 1.39 g (84%); yellow foam; R_f = 0.54 (CH_2Cl_2–MeOH, 85:15).

^{1}H NMR (200 MHz, DMSO-d_6): δ = 1.87–2.21 [m, 2 H, C(β)H_2, Glu], 2.46 [t, ^3J = 7.5 Hz, 2 H + DMSO-signal, C(γ)H_2, Glu], 3.09 (s, 3 H, NCH_3), 3.23 (s, 3 H, NCH_3), 3.60 (s, 3 H, OCH_3), 3.67 (s, 3 H, OCH_3), 4.46 [m, 1 H, C(α)H, Glu], 5.26 (s, 2 H, H-6), 7.43 (dd, ^3J = 8.4 Hz, ^4J = 2.1 Hz, 1 H, 5′-H_{arom}), 7.57–7.67 (m, 2 H, 6′-H_{arom}, 3′-H_{arom}), 8.65 (dd, ^3J = 7.5 Hz, ^4J = 1.4 Hz, 1 H, NH, Glu), 8.74 (s, 1 H, H-7), 8.79 (s, 1 H, CH=N), 8.87 (s, 1 H, CHO), 11.99 [br s, 1 H, N(3)H].

^{13}C NMR (50 MHz, DMSO-d_6): δ = 25.7, 29.7, 34.9, 40.9, 46.7, 51.3, 51.8, 52.0, 109.3, 109.8, 117.3, 120.0, 120.3, 129.8, 130.9 (d), 144.4, 144.6, 146.7, 148.7, 155.5, 157.1, 158.2, 161.5, 171.7, 172.6.

HRMS (ESI, MeOH-CH_2Cl_2): *m*/*z* [M + H]$^+$ calcd for $C_{25}H_{28}FN_8O_7$: 571.2059; found: 571.2054.

Acknowledgment

We thank Michael Blatti, Thomas Ammann, and Katja Rohmer for technical assistance.

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