SUPPLEMENTARY INFORMATION

MANUSCRIPT

Specific PET imaging of x_C^- transporter activity using a ^{18}F-labeled glutamate derivative reveals a dominant pathway in tumor metabolism

AUTHORS

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METHODS & RESULTS

1. Chemistry

1.1. General Methods

^{1}H NMR and ^{13}C NMR data were obtained on a Bruker DRX 600 NMR spectrometer with $CDCl_3$ as solvent and tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in parts per million (ppm, δ scale) by assigning TMS resonance in the ^{1}H NMR spectrum as 0.00 ppm and the carbon resonances of the NMR solvent ($CDCl_3$) as δ 77.0. Data were reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, quin = quintet, m = multiplet and br = broad), coupling constant (J values) in Hz and integration. The ESI mass spectra (positive ions, acetonitrile/water acidified with 0.05% formic acid) were measured on Waters Platform SQD.

Flash column chromatography was performed using 200-300 mesh silica with the indicated solvent system according to standard techniques. Analytical thin-layer chromatography (TLC) was performed on pre-coated, glass-backed silica gel plates.

Di-*tert*-butyl N-(*tert*-butoxycarbonyl)-L-glutamate was prepared as described previously.[1] All other reagents were obtained from commercial sources and used as received.

Radiochemical purity of (4*S*)-4-[3-[^{18}F]fluoropropyl]-L-glutamate was determined by Thin Layer Chromatography (TLC) and High Pressure Liquid Chromatography (HPLC).

<u>TLC method</u>

Stationary phase:	silica gel (Mesh 60) on aluminum foil $60F_{254}$, Merck
Mobile phase:	*n*-BuOH / AcOH / water / EtOH (12 / 3 / 5 / 1.5)
Detection:	Phosphorimager SI Molecular Dynamics

<u>HPLC method 1</u>

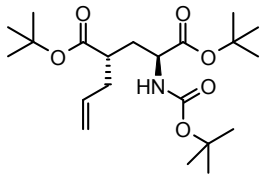
Equipment:	Agilent 1100
Column:	Hypercarb 7µ; 100x4.6 mm; ThermoHypersil
Flow:	2.0 mL/min
Eluent:	MeCN/water (+ 0.1% TFA); 2% MeCN
Detectors:	Agilent 1100, DAD G1315B (UV); Corona detector (ESA Biosciences); Raytest Gabi (γ)

HPLC method 2 – pre column derivatization

Equipment:	Agilent 1100 / Agilent 1200
Column:	Luna 5µ C18(2); 250*4,6mm; 5µ; Phenomenex
Flow:	1.2 mL/min
Eluent:	12% MeCN in 10 mM Na_2HPO_4
Detectors:	Agilent 1200, DAD G1315C (UV); Corona detector (ESA Biosciences); Raytest Gabi (γ)
Derivatization:	10 µL sample were mixed with 30 µL OPA-reagent (Fluoraldehyde™ *o*-Phthalaldehyde Reagent Solution; Thermo Scientific). Mixing was done manually or by means of the autosampler of the HPLC.

1.2. Synthesis of di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-nitrophenylsulfonyloxy-propyl)-pentanedioate[2]

Step 1: Preparation of Di-*tert*-butyl (2*S*,4*S*)-4-allyl-2-tert-butoxycarbonylamino-pentanedioate



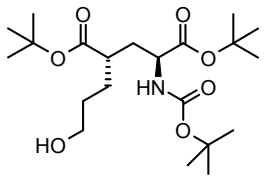
Di-*tert*-butyl (4*S*)-4-allyl-*N*-(*tert*-butoxycarbonyl)-L-glutamate was synthesized analogous to Belvisi et al.[3] 26.96 g (75 mmol) of di-*tert*-butyl Boc-glutamate[1] were dissolved in 220 mL of tetrahydrofuran (THF) and cooled to -70°C. 165 mL (165 mmol) of a 1M solution of lithium bis(trimethylsilyl)amide in THF were added dropwise over a period of two hours at this temperature and the mixture was stirred at -70°C for another 2 hours. 27.22 g (225 mmol) of allyl bromide were then added dropwise, and after 2 h at this temperature, the cooling bath was removed and 375 mL of 2N aqueous hydrochloric acid and 1.25 L of ethyl acetate were added. The organic phase was separated off, washed with water until neutral, dried over sodium sulphate and filtered, and the filtrate was concentrated. The crude product was purified by flash chromatography (hexane/ethyl acetate = 97/3 to 85/15) and the appropriate fractions were combined and concentrated.

Di-tert-butyl (4*S*)-4-allyl-*N*-(*tert*-butoxycarbonyl)-L-glutamate was isolated as colorless oil (15.9 g, 53.1%).

MS (ESIpos): m/z = 400 $[M+H]^+$

^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ ppm 1.32-1.58 (m, 27H) 1.81-1.92 (m, 2H) 2.25-2.39 (m, 2H) 2.40-2.48 (m, 1H), 4.10-4.18 (m, 1H) 4.85-4.92 (d, 1H) 5.02-5.11 (m, 2H) 5.68-5.77 (m, 1H)

Step 2: Preparation of di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-hydroxypropyl)-pentanedioate

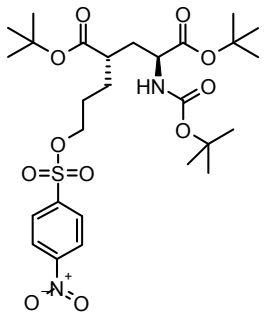


15,58 g (39 mmol) of di-*tert*-butyl (2*S*,4*S*)-4-allyl-2-tert-butoxycarbonylamino-pentanedioate were dissolved in 200 mL of tetrahydrofuran and cooled in an ice-bath. Over a period of 20 minutes, 54.6 mL (54.6 mmol) of 1 M diborane/tetrahydrofuran complex in tetrahydrofuran were added dropwise with ice-cooling and under nitrogen, and the mixture was stirred on ice for 2 h and at room temperature overnight. It was cooled again to 0°C and 58.5 mL of 1 N aqueous sodium hydroxide solution and 58.5 mL of 30% aqueous hydrogen peroxide solution were then added dropwise. After 30 minutes, the mixture was diluted with water, the tetrahydrofuran was distilled off and the remaining aqueous solution was extracted with ethyl acetate. The organic layer was washed with water and two times with NaCl solution. After drying over sodium sulfate, the solvent was evaporated. The crude product was purified by flash chromatography (hexane/ethyl acetate = 92/8 to 35/65).
Di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-hydroxypropyl)-pentanedioate) was isolated as colorless oil (8.5 g, 52.2 %).

MS (ESIpos): m/z = 418 $[M+H]^+$

^{1}H NMR (300 MHz, CHLOROFORM-*d*) δ ppm 1.32-1.58 (m, 27H) 1.60-1.70 (m, 2H) 1.73-1.94 (m, 4H) 2.05-2.12 (m, 1H), 2.33-2.40 (m, 1H) 3.58-3.68 (m, 2H) 4.15-4.22 (m, 1H) 4.95-5.03 (d, 1H)

Step 3: Preparation of di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-nitrophenylsulfonyloxy-propyl)-pentanedioate



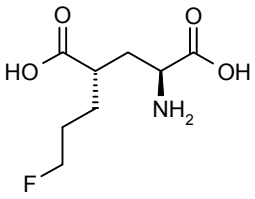
5.22 g (12.5 mmol) of di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-hydroxypropyl)-pentanedioate was dissolved in 125 mL of dichloromethane and cooled in an ice-bath. After addition of 7.59 g (75 mmol) of triethylamine and 5.54 g (25 mmol) nitrophenylsulfonyl chloride, the mixture was stirred on ice for 2 h. The solvent was then removed under reduced pressure and the crude product was purified by flash chromatography (hexane/ethyl acetate). Di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-nitrophenylsulfonyloxy-propyl)-pentanedioate was isolated as colorless oil (4.7 g, 62.4 %).

MS (*m/z*): $[M+H]^+ = 603$; M = $C_{27}H_{42}N_2O_{11}S$

^{1}H-NMR (600MHz, CHLOROFORM-d): δ[ppm]= 1.42 (s, 9H, *t*Bu), 1.43 (s, 9H, *t*Bu), 1.45 (s, 9H, *t*Bu), 1.57-1.66 (m, 2H, C_γ-CH_2), 1.66-1.75 (m, 2H, $CH_2C\mathit{H}_2CH_2$), 1.75-1.87 (m, 2H, Glu-$C_ßH_2$), 2.29 (quin, 1H, $C_\gamma H$), 4.07-4.12 (m, 1H, $C_\alpha H$), 4.13-4.16 (m, 2H, CH_2-OSO_2), 4.86 (m,1H, NH), 8.12 (d, 2H, Ar-CH), 8.42 (d, 2H, Ar-CH).

^{13}C-NMR (600MHz, CHLOROFORM-d): δ [ppm]= 26.42 (CH_2, $CH_2\mathit{C}H_2CH_2$), 27.87 (CH_2, C_γ-$\mathit{C}H_2$), 27.95 (CH_3, *t*Bu), 28.03 (CH_3, *t*Bu), 28.26 (CH_3, *t*Bu), 34.52 (CH_2, $C_ß$), 42.64 (CH, C_γ), 52.85 (CH, C_α), 71.26 (CH_2, CH_2-OSO_2), 79.82 (C_q, *t*Bu), 81.11 (C_q, *t*Bu), 82.13 (C_q, *t*Bu), 124.49 (CH, C_{aryl}), 129.22 (CH, C_{aryl}), 141.88 (C_q, C-SO_2), 150.76 (C_q, C-NO_2), 155.53 (C, Boc-CO), 171.58 (C, *C*OO*t*Bu), 174.20 (C, *C*OO*t*Bu),

1.3. Synthesis of (4*S*)-4-(3-Fluoropropyl)-L-glutamate (^{19}F-BAY 94-9392)



To 45.93 g (110 mmol) of di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-hydroxypropyl)-pentanedioate in 1100 mL THF were added 66.79 g (92 mL, 660 mmol) of triethylamine. After addition of 66.46 g (39.56 mL, 220 mmol) of perfluorobutan-1-sulfonyl fluoride and of 35.57 g (35.86 mL, 220 mmol) triethylamine/$(HF)_3$ complex the mixture was stirred 2 d at room temperature. The solvent was then removed under reduced pressure and the crude product was purified by flash chromatography (75 L cartridge, Biotage) using a hexane/ethyl acetate gradient. (2*S*,4*S*)-2-*tert*-Butoxycarbonylamino-4-(3-fluoro-propyl)-pentanedioic acid di-*tert*-butyl ester was isolated as colorless oil (25 g, 54.2 %). The resulting oil was dissolved slowly in 162 mL (2.1 mol) trifluoro acetic acid at 0° C and stirred for 3 d at room temperature. After evaporation the residue was taken up in diethyl ether and evaporated again. This procedure was repeated twice and the solid residue was then stirred in 500 mL of diethyl ether overnight, filtered off and taken up in 250 mL of water. After addition of approx. 57 mL (57 mmol) 1N NaOH the pH was adjusted to 7.4 and the aqueous solution was lyophilized. The amorphous powder obtained was absorbed on 12 g silica gel and was chromatographed on Lichroprep RP 18 (500 g; 40-63 µm) using a water/acetonitrile gradient (100/0 to 0/100).

(4*S*)-4-(3-Fluoropropyl)-L-glutamate was isolated after lyophilization as a colorless amorphous powder (3 g, 26.8 %).

MS (*m/z*): $[M+H]^+ = 208$; M = $C_8H_{14}FNO_4$

^{1}H-NMR (600MHz, METHANOL-d_4): δ[ppm]= 1.62-1.80 (m, 4H, FCH_2-C*H*$_2$C*H*$_2$), 1.87 (dt, 1H, Glu-C$_ß$H), 2.11 (dt, 1H, Glu-C$_ß$H), 2.47-2.52 (m, 1H, C$_γ$H), 3.45 (dd, 1H, C$_α$H), 4.41 (dt, 2H, FCH_2).

^{13}C-NMR (600MHz, METHANOL-d_4): δ [ppm]= 29.23 (CH_2, CH_2*C*H_2CH_2), 30.47 (CH_2, C$_γ$-*C*H_2), 35.30 (CH_2, C$_ß$), 48.13 (CH, C$_γ$), 56.56 (CH, C$_α$), 84.90 (CH_2, CH_2F), 174.98 (C, C$_α$-*C*OO), 182.66 (C, C$_γ$-*C*OO).

1.4. Synthesis of (4*S*)-4-[3-[^{18}F]fluoropropyl]-L-glutamate (BAY 94-9392)

The synthesis of (4*S*)-4-[3-[^{18}F]fluoropropyl]-L-glutamate was performed on a remotely controlled synthesis module (Eckert&Ziegler modular lab). [^{18}F]fluoride was trapped on an anion exchange cartridge (QMA SepPak light, Waters). The activity was eluted with kryptofix/potassium carbonate solution (5 mg kryptofix, 1 mg K_2CO_3 in 1 mL water/acetonitrile) into the reaction vessel. The mixture was dried (120 °C, nitrogen stream, vacuum). Drying was repeated after addition of 1 ml acetonitrile. 5 mg nosylate precursor (Di-*tert*-butyl (2*S*,4*S*)-2-*tert*-butoxycarbonylamino-4-(3-nitrophenylsulfonyloxy-propyl)-pentanedioate) in 1 mL acetonitrile were added to the dried residue and the resulting solution was stirred for 1-10 min at 60-80 °C. 2N HCl (2 mL) was added and the mixture was heated at 100 °C for 5 min. After cooling to 60 °C, the solution was diluted with water (50 mL) and passed through a HR-P (Chromafix HR-P, Macherey-Nagel) and a MCX cartridge (Oasis MCX 20cc (1 g), Waters). The MCX cartridge was washed with saline (20 mL) and (2*S*,4*S*)-4-{3-[^{18}F]fluoropropyl}-glutamic acid was eluted with 10 mL buffer (70 mg $Na_2HPO_4 \cdot 2H_2O$, 60 mg NaCl in 10 mL water) into the product vial. 40-63 % (corrected for decay) (2*S*,4*S*)-4-{3-[^{18}F]fluoropropyl}-glutamic acid were obtained in 41-50 min overall synthesis time. Identity was proven by co-elution with reference standard (4*S*)-4-(3-Fluoropropyl)-L-glutamic acid) and radiochemical purity was analyzed by Radio-TLC (R_f = 0.46), HPLC Method 1 (R_t = 2.7 min) and HPLC Method 2 (R_t = 13.5 min) and determined to be > 92%.

2. Biology

2.1. Proliferation

Growth curves of A549 cells transduced with lentivirus plasmids harbouring xCT shRNA (sh-1487 and sh-7701) and non-silencing shRNA(sh-QIA control) were recorded. Seven days after transduction the cells were seeded in a 96-well plate. Cell growth was monitored by crystal violet staining (absorbance at 595 nm). The proliferation rate for the xCT knock-down clones #1487 and #7701 was reduced to 44 - 56 % compared to mock treated A549 cells.

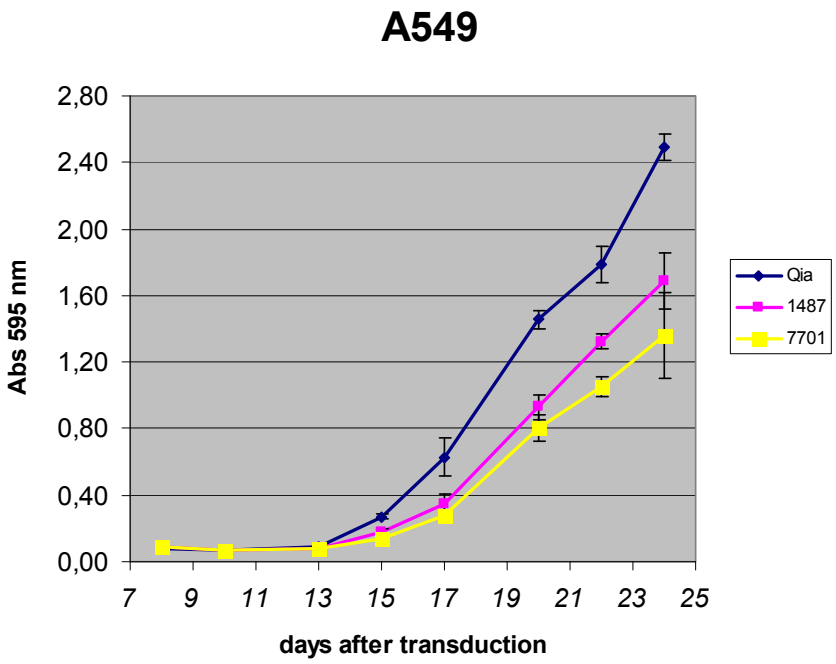


Figure S1: shRNA-mediated xCT silencing reduces proliferation of A549 human lung adenocarcinoma cells. Proliferation rate was monitored by crystal violet staining.

2.2. In vivo comparison BAY 94-9392 and FDG

BAY 94-9392 (16 MBq) was administered via tail vein injection in NCI-H460 human lung tumor (NSCLC) bearing nude rats. PET imaging was performed at 90 min p.i. with 20 min scan time. Data were acquired in a single bed position (thorax to lower extremities, full axial field of view 12.7 cm).

A comparative PET study was performed using BAY 94-9392 and FDG in NCI-H460 bearing nude rats. BAY 94-9392 and FDG were studied with the same animal handling and imaging protocol. The tumor was clearly visualized with BAY 94-9392 (figure S2, left)and FDG (figure S2, right). In addition to tumor uptake of FDG a significant tracer uptake was observed in muscle tissue, joints, brown fat and heart.

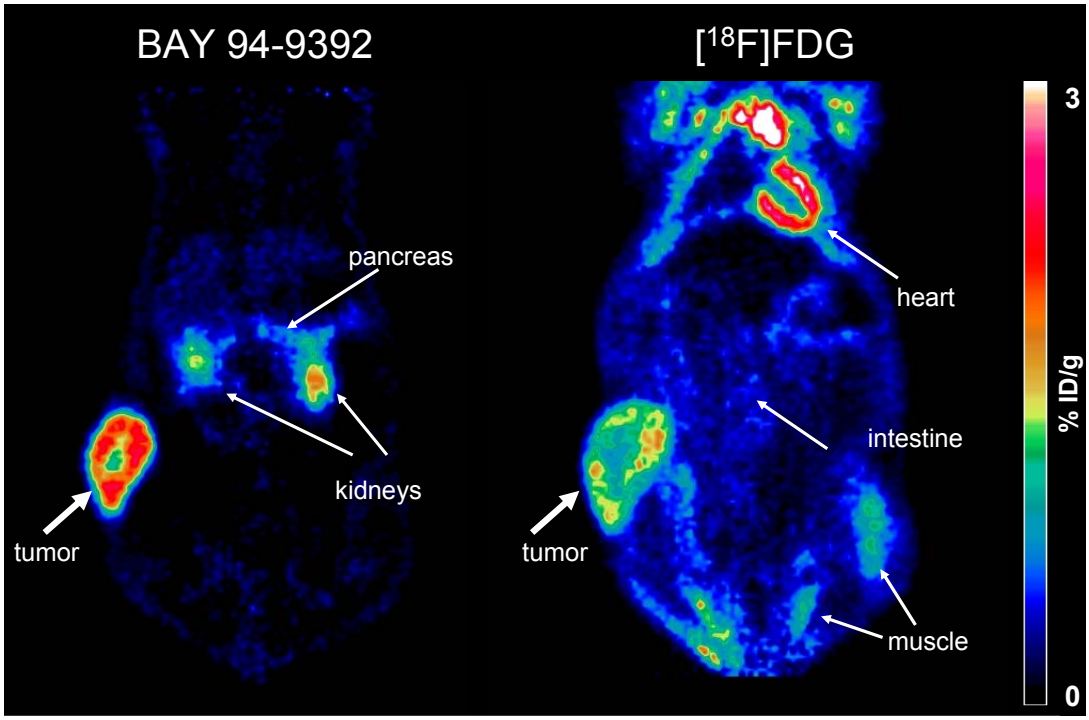


Figure S2: Direct comparison of BAY 94-9392 and FDG in the same NCI-H460 tumor rat using the identical animal handling and imaging protocol. PET study with BAY 94-9392 was performed 10 days after tumor cell inoculation, the FDG PET scan was performed at day 12 after inoculation. Data acquisition was started 90 min p.i. for 20 min. Representative coronal sections are shown

2.3. *In vivo* stability of BAY 94-9392

A stability study was performed in mice after intravenous application of BAY 94-9392 (n = 3 per time point). 15 MBq of BAY 94-9392 in 150 µl PBS buffer were injected per animal. Animals were sacrificed at 10, 20 and 30 min p.i. and blood was collected in K/EDTA vials. Blood plasma was obtained by centrifugation (5 min, 3000 rpm): Plasma proteins were removed by centrifugation using centrifugal filters with a 30 kDa cut-off (VWR International). The filtrate was analyzed by thin-layer chromatography (TLC). Silica plates (PLC Silica gel 60 F254, 2 mm with concentrating zone (Merck KGaA, Darmstadt, Germany) were used with *n*-Butanol/H_2O/AcOH/EtOH (12 / 5 / 3 / 1.5) as solvent. 10 µL were spotted and the TLC plates were developed for 60 min. Subsequently, TLC plates were exposed to phosphorimager plates for 2 h, scanned with the phosphorimager (Storm 825, General Electric) and analyzed with software provided by the manufacturer.

Figure S3 shows the scanned phosphorimager plate. Blood samples from the 10 min time point (n=2) and blood samples from the 20 min and 30 min time point (n=3) were spotted on the TLC plate. No signals different from BAY 94-9392 (R_f = 0.7) were detected in the plasma samples, indicating high *in vivo* stability of BAY 94-9392 in mice up to 30 min. Later time points were not studied due to the rapid blood clearance.

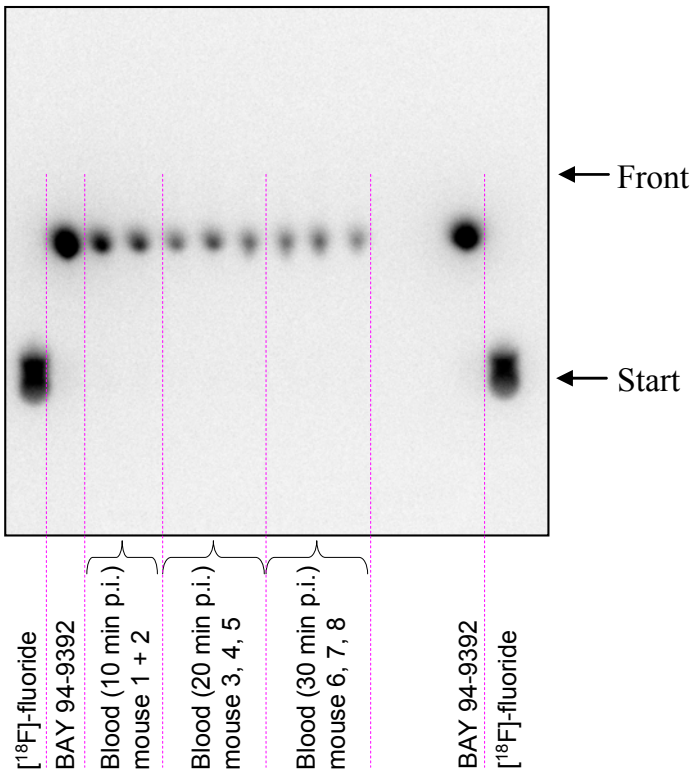


Figure S3: Analysis of the *in vivo* stability of BAY 94-9392 after intravenous injection in NMRI mice. Only the parent compound (R_f = 0.7) and no defluorination were detected in blood plasma samples up to 30 min post injection.

3. References

[1] Han, G., Tamaki, M. & Hruby, V. J. Fast, efficient and selective deprotection of the tert-butoxycarbonyl (Boc) group using HCl/dioxane (4 M). *J. Peptide Res*. **58**, 338-341 (2001).

[2] Berndt M, Schmiitt-Willich H, Friebe M, Graham K, Brumby T Hultsch C, et al. Method for production of F-18 labeled glutamic acid derivatives (Bayer Schering Pharma Aktiengesellschaft), WO 2011 / 060887 A2.

[3] Belvisi, L. *et al.* Practical stereoselective synthesis of conformationally constrained unnatural proline-based amino acids and peptidomimetics, *Tetrahedron* **57**, 6463-6473 (2001).