Development of the First Aliphatic ^{18}F-Labeled Tetrazine Suitable for Pretargeted PET Imaging – Expanding the Bioorthogonal Tool Box

Umberto Maria Battisti[†a], Klas Bratteby[†a,b,c], Jesper T. Jørgensen[d], Lars Hvass[d], Vladimir Shalgunov[a], Hannes Mikula[e], Andreas Kjaer[d], and Matthias Manfred Herth[a,b,*]

[a]*Department of Drug Design and Pharmacology, Faculty of Health and Medical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark.*

[b]*Department of Clinical Physiology, Nuclear Medicine & PET, Rigshospitalet, Blegdamsvej 9, 2100 Copenhagen, Denmark.*

[c]*Department of Radiation Physics, Skåne University Hospital, Barngatan 3, 22242 Lund, Sweden*

[d]*Cluster for Molecular Imaging, Department of Biomedical Sciences, University of Copenhagen, Blegdamsvej 3, 2100 Copenhagen Ø, Denmark.*

[e]*Institute of Applied Synthetic Chemistry, Technische Universität Wien (TU Wien), Getreidemarkt 9, 1060 Vienna, Austria.*

Abstract

Pretargeting imaging of nanomedicines have attracted considerable interest in nuclear medicine since it has the potential to increase imaging contrast while simultaneously reducing radiation burden to healthy tissue. Currently, the tetrazine ligation is the fastest bioorthogonal reaction available for this strategy and consequently, the state-of-art choice for *in vivo* chemistry. We have recently identified key properties for tetrazines to be applied in pretargeting. We have also developed a method to ^{18}F-label highly reactive tetrazines using an aliphatic nucleophilic substitution strategy. In this study, we combined this knowledge and developed an ^{18}F-labeled tetrazine for pretargeted imaging. In order to develop this ligand, a small structure-property study was carried out. The most promising compound - with respect to reactivity, hydrophilicity and *ex vivo* blocking effect - was selected for labeling and subsequent PET *in vivo* imaging. Radiolabeling was achieved in satisfying radiochemical yields, molar activities as well as in high radiochemical purities. The tracer displayed favorable pharmacokinetics and remarkable target-to-background ratios in pretargeted experiments - already one hour post injection. We believe that the developed pretargeting imaging agent is a promising candidate for translation into clinical studies.

Keywords*: bioorthogonal chemistry, tetrazine ligation, pretargeted imaging, PET, fluorine-18, molecular imaging*

Introduction

Nanomedicines such as monoclonal antibodies or other nanoparticles have received increased interest e.g. in the field of oncology and drug delivery within the last decades.[1-3] They can be used as selective drug or radionuclide delivery vectors.[4, 5] Their unique targeting properties allow for specific accumulation up to 30-50% injected dose per kg (ID/kg) in patients, and as such, hold great potential to become state-of-the-art treatment.[5] To identify which patients would benefit from nanomedicines, the targeting abilities of each nanomedicine needs to be quantified on an individual basis (precision medicine).[6-8] Patient-to-patient variations in tumor uptake, even within the same cancer type, represent a challenge to identify the optimal therapeutic dose.[6-8] This is of particular interest for radionuclide therapies, where radiolabeled compounds are designed with the aim of delivering radiation doses to specific targets upon injection.[6-8] Molecular imaging techniques are commonly applied to estimate the maximum tolerated radiation dose of such therapies, in respect to highest effectiveness with tolerable site-effects (theranostic concept) (Figure 1A). [6-8] Nanomedicines usually possess slow pharmacokinetics, i.e. slow target accumulation as well as slow excretion. These processes can take days to weeks.[9, 10] For targeted radionuclide approaches, this displays a challenge since high radiation doses are then delivered to healthy tissues, which limits and often prohibits the clinical application of these compounds.[9, 10]

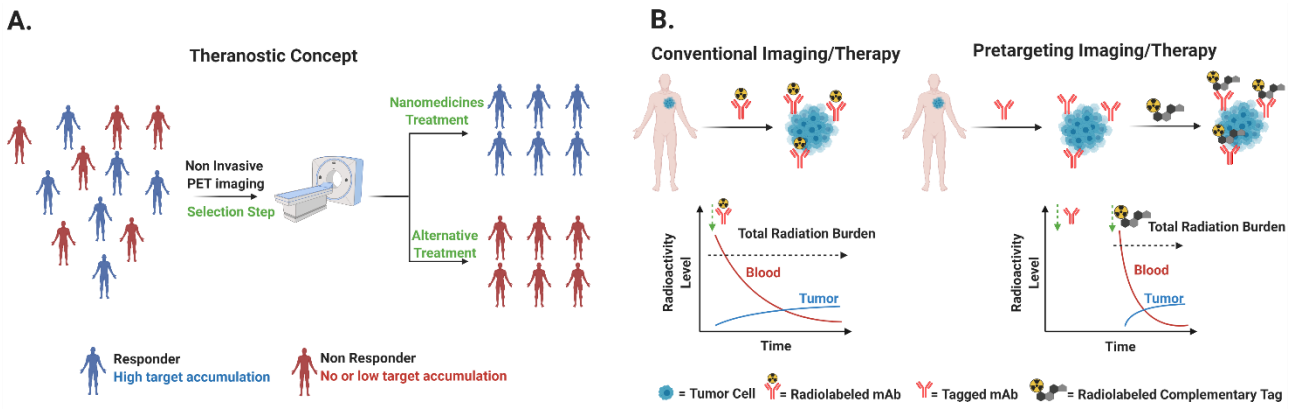


Figure 1. Application of nanomedicines for precision medicine; **A**. Theranostic concept: with imaging, possible responders to a certain therapy can be identified. If identified, the therapy can be initiated. Otherwise, an alternative therapeutic strategy should be used **B**. Comparison between conventional imaging/therapy *vs* pretargeted imaging/therapy. Pretargeted strategies result in lower radiation burden to healthy tissue, here exemplified by difference in the blood compartment. The imaging/radionuclide therapy is initiated when the nanomedicine has already accumulated at the target and the radiolabeled complementary tag is excreted at a much higher rate than the nanomedicine.

Pretargeting offers an intriguing alternative which circumvent the dose limitations that conventional nanomedicine-based radionuclide therapies possess.[11, 12] Pretargeted strategies allow to label nanomedicines when they have already reached their target and have cleared from the rest of the body.[11-14] The targeting nanomedicine is modified with a bioorthogonal tag and injected. The nanomedicine is allowed to accumulate at the target and to clear from the rest of the body.

Subsequently, a complementary tag is radiolabeled and administered. This tag will bioorthogonally react with the tagged nanomedicine *in vivo* – conceptionally only at the target site and within minutes while unreacted tags are excreted rapidly. Thus, good target-to-background ratios can already be obtained after minutes (Figure 1B). Consequently, radiation dose to healthy tissue is minimized.[6, 11, 15, 16] A number of different bioorthogonal reactions have been employed for such approaches.[11, 15, 17, 18] Currently, the tetrazine ligation between a tetrazine (Tz) and a *trans*-cyclooctene (TCO) is the most effective reaction in this respect.[19-22] From a clinical point of view, fluorine-18 is an almost ideal radionuclide for Positron-Emission-Tomography (PET).[23-25] Its unique decay characteristics give rise to high resolution PET images and result in acceptable levels of radiation burden. In addition, the half-life of 110 min also enables distribution from the production site to other research facilities and clinics, and allows, as such, for commercialization.[23-25] We have recently reported the first successful direct labeling of ^{18}F-Tzs with suitable reactivity for *in vivo* chemistry.[26] In another study, we identified key properties for Tzs to be used for pretargeted strategies.[25] In this study, we combined this knowledge and developed the first direct aliphatic ^{18}F-radiolabeled Tz suitable for pretargeted PET imaging. This tracer could potentially be used for dose estimations before pretargeted radionuclide approaches are initiated.

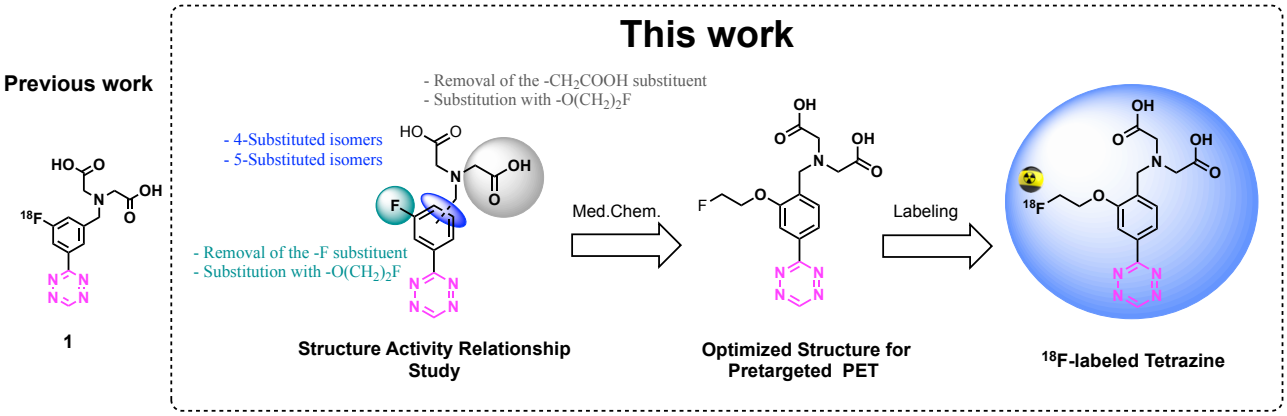


Figure 2. Design strategy to develop an aliphatic ^{18}F-Tz suitable for *in vivo* chemistry. The starting point for our structure activity relationship study was based on compound **1** - a recently reported successful pretargeted imaging agent. Our aim was to develop an agent without the use of a Cu-mediated tin-precursor and thus increase the clinical translatability.

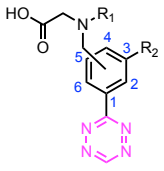
Result and Discussion

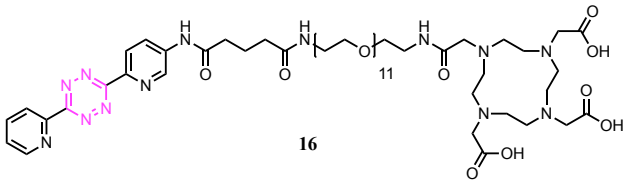
Design Strategy

Compound **1** was selected as a starting point for the design of new tracers. This compound was designed in accordance with our findings that high reactivity (>50.000 $M^{-1}s^{-1}$ with TCO in PBS) as well as low lipophilicity ($cLogD_{7.4}$ <-3) determine the *in vivo* ligation ability of tetrazines for pretargeted strategies.[25] Indeed, [^{18}F]**1** showed good tumor-to-background ratios *in vivo* – already one hour after application.[26] However, radiolabeling of [^{18}F]**1** is based on a Cu-mediated strategy using a tin-precursor. Consequently, clinical translation of this compound might be hampered as the Cu- and

Sn-content of the final formulation must be validated to be below permission limits – presumably for every production. In order to circumvent these challenges, we want to exploit our latest labeling strategy, which permits aliphatic labeling of highly reactive H-Tz derivatives.[27] For this reason, we evaluated how to introduce a fluoroethyl moiety into the base structure of **1**, while retaining the necessary key properties for *in vivo* chemistry. We explored how **a**) the removal of one acetic acid chain, **b**) the introduction of a fluoroethyl moiety, **c**) the removal of the -F atom on the aromatic ring and **d**) varying the substitution pattern of the phenyl ring, influences the possibility of tetrazines to ligate *in vivo* (Figure 2). In this respect, we designed 15 compounds (Table 1).

Table 1. Structural scaffolds, calculated physicochemical properties (TPSA, clog$D_{7.4}$), measured second-order rate constants for the IEDDA reaction with TCO, and blocking efficiencies of all investigated tetrazine derivatives.





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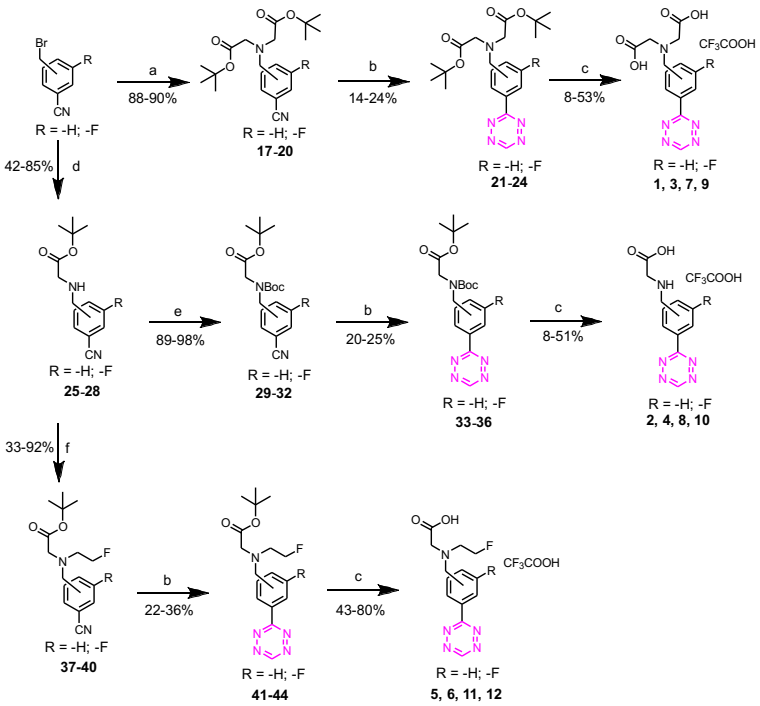
Tetrazine[a]	Side Chain Position	R_1	R_2	ClogD$_{7.4}$[b]	TPSA[c]	Rate constant (M^{-1} s^{-1})[d]	Blocking effect [g]	% Tumor Uptake of [^{111}In]16 after blocking
1[a]	5	-CH_2COOH	-F	-6.93	129.40	82000	90[h]	10[h]
2[a]	5	-H	-F	-2.89	100.89	78000	95	5
3[a]	5	-CH_2COOH	-H	-6.97	129.40	62000	95	5
4[a]	5	-H	-H	-3.03	100.89	55000	96	4
5[a]	5	-CH_2CH_2F	-F	-2.67	92.10	88000	76	24
6[a]	5	-CH_2CH_2F	-H	-2.73	92.10	62000	75	25
7[a]	4	-CH_2COOH	-F	-6.91	129.40	76000	98	2
8[a]	4	-H	-F	-2.98	100.89	76000	98	2
9[a]	4	-CH_2COOH	-H	-6.98	129.40	62000	97[i]	3[i]
10[a]	4	-H	-H	-3.03	100.89	60000	86[i]	14[i]
11[a]	4	-CH_2CH_2F	-F	-2.77	92.10	74000	72	28
12[a]	4	-CH_2CH_2F	-H	-2.73	92.10	55000	69	31
13[a]	4	-CH_2COOH	-OH	-7.43	149.63	n.d.	99	1
14[a]	4	-CH_2COOH	-OCH_3	-7.24	138.63	68000	99	1
15[a]	4	-CH_2COOH	-OCH_2CH_2F	-6.83	138.63	68000	98	2
16[e]	-	-	-	-4.13[f]	358.03	74000	99	1

Notes: [a] The compounds were obtained as trifluoroacetate salt. [b] Calculated distribution coefficient at physiological pH (7.4) in Chemicalize software. [c] Calculated in Chemicalize software. [d] Second order rate constants are determined with TCO-PEG4 (modified TCO-5ax−OH, "minor-TCO") in Dulbecco's phosphate buffered saline (DBPS) at 37°C (see supporting information for details).[e] The compound was employed as a reference. [f] Calculated as chelated to a trivalent cation. n.d. = not determined. [g] The blocking effect of non-radiolabeled Tz was determined as the change in tumor uptake of [^{111}In]**16** 22 h p.i. Each Tz was administered 1 h prior to [^{111}In]**16**, and the uptake was normalized to a group of animals in which no blocking was performed (control). Data represent mean from n = 3 mice/group, detailed information can be found in the Experimental Part. [h] Blocking data from ref. 26. [i] Blocking data from ref. 25.

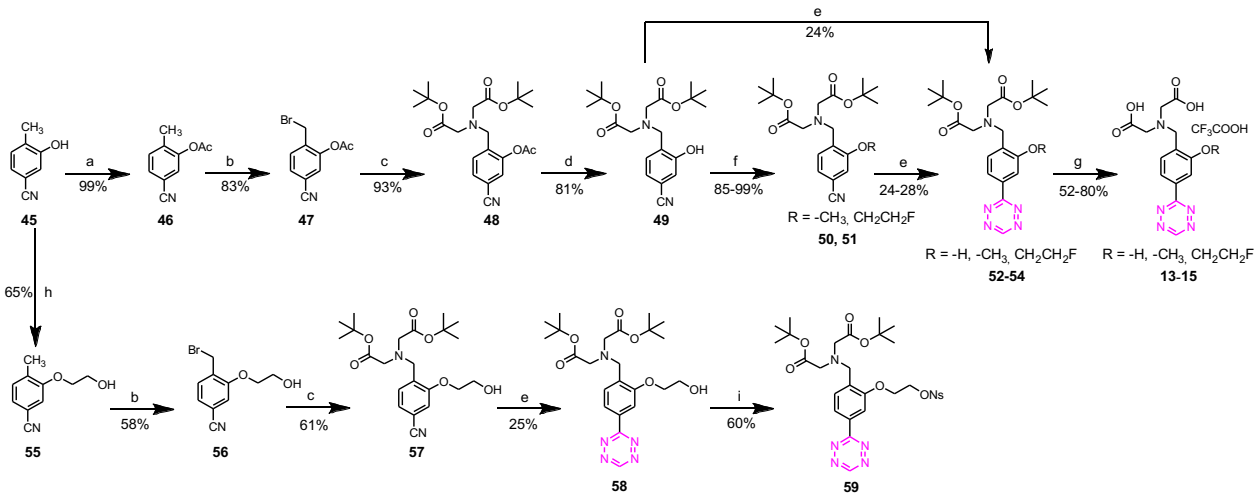
Reference Compounds Synthesis

The key step within the synthesis of all reference compounds was the formation of the H-phenyl-tetrazine core. It was synthesized using a Pinner-like, sulfur-mediated procedure reported by Qu et al. in modest yields up to 36%.[28] This procedure is reported to result in higher yields for H-phenyl-tetrazines compared to "standard" or alternative Pinner-like procedures.[29-31] The proposed mechanism for the sulfur-mediated version is based on the formation of the reactive nucleophile NH_2NHSH, which reacts then more efficiently - compared to hydrazine used typically in Pinner-like reactions - with respective nitriles to form dihydrotetrazines under H_2S elimination. Dihydrotetrazines can then easily be oxidized (e.g. by $NaNO_2$) to their correspondent tetrazines. *N*-benzyliminodiacetic acid derivatives **1**, **3**, **7**, **9** were obtained starting from the corresponding benzyl bromide derivative which were either commercially available or could be synthesized via radical bromination of their toluene derivatives (Scheme 1). Subsequent tetrazine formation, deprotection with trifluoroacetic acid (TFA) and preparative high-performance liquid chromatography (HPLC) purification afforded the desired Tzs. Monoacetic acid derivatives **2**, **4**, **8** and **10** were synthesized in a similar manner (Scheme 1). Alkylation of *tert*-butyl protected glycine and subsequent Boc protection gave the required nitrile derivatives in satisfying yields. Tetrazine formation and deprotection resulted as well in the desired products. Reaction of *tert*-butyl glycine nitrile derivatives with 1-fluoro-2-iodoethane yielded in the necessary intermediates to obtain **5**, **6**, **11** and **12**, which were synthesized with a similar strategy to the one reported above (Scheme 1). Compound **13-15** were obtained likewise (Scheme 2). Protection of the phenolic group and radical bromination followed by alkylation gave compound **49**.



Scheme 1. *Reagents and conditions:* a) di-*tert*-butyl iminodiacetate, K_2CO_3, CH_3CN, rt, 12 h; b) *i*) $NH_2NH_2{\cdot}H_2O$, CH_2Cl_2, S_8, EtOH, 50 ºC, 24 h; *ii*) $NaNO_2$, AcOH, 0 ºC to rt, 30 min; c) TFA, CH_2Cl_2, rt, 2 h; d) glycine *tert*-butyl ester hydrochloride, K_2CO_3, CH_3CN, rt, 12 h; e) Boc_2O, Et_3N, CH_2Cl_2, rt, 12 h; f) 1-fluoro-2-iodoethane, K_2CO_3, CH_3CN, reflux, 24 h.

The latter was deprotected under basic conditions and further derivatized to give compounds **50** and **51**. Subsequent tetrazine formation, deprotection with TFA and preparative HPLC purification afforded the desired Tzs **13-15**.



Scheme 2. *Reagents and conditions:* a) Ac_2O, Et_3N, CH_2Cl_2, rt, 12 h; b) *N*-bromosuccinimide; AIBN, $CHCl_3$, reflux, 12 h; c) di-*tert*-butyl iminodiacetate, Et_3N, CH_3CN, rt, 12 h; d) NaOH, H_2O, CH_3CN, rt, 1 h; e) *i*) $NH_2NH_2{\cdot}H_2O$, CH_2Cl_2, S_8, EtOH, 50 ºC, 24 h; *ii*) $NaNO_2$, AcOH, 0 ºC to rt, 30 min; f) CH_3I or 1-fluoro-2-iodoethane, K_2CO_3, CH_3CN, reflux, 24 h; g) TFA, CH_2Cl_2, rt, 2 h; f) 2-bromoethanol, NaOH, H_2O, reflux 24 h; i) Nosyl chloride, DIPEA, DMAP, CH_2Cl_2, rt, 6 h.

Reactivity of the Tz Library

Second-order rate constants for the reaction with TCO in PBS at 37 °C were determined by pseudo-first order measurements in a SX20 stopped flow photometer (Applied Photophysics). All tetrazines displayed rate constant values >55.000 $M^{-1}s^{-1}$ and are as such within the limit of suggested values that increase the chance of a tetrazine to be used for pretargeted *in vivo* chemistry (Table 1).[25]

Ex vivo Blocking Assay

We have recently developed a blocking assay that allows to assess the *in vivo* ligation performance of unlabeled tetrazine derivatives. This assay omits time-consuming development of radiolabeled tetrazines for every ligand to be tested. It is based on the ability of tetrazines to block the binding of pretargeted imaging agent [^{111}In]**16,** to the pretargeting vector CC49-TCO (administered 72 h prior) in tumor bearing mice. The setup has previously been described in literature.[25] The tumor blocking effect of the unlabeled tetrazine derivatives is afterwards determined by *ex vivo* biodistribution and normalized to the binding of [^{111}In]**16** without any blocking. The setup is displayed in Figure 3.

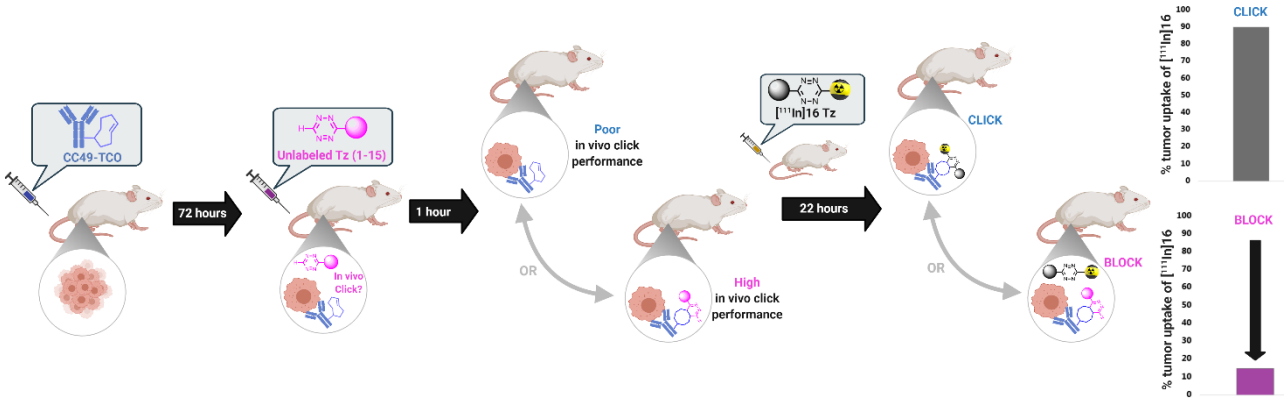


Figure 3. Schematic display of the blocking assay. The blocking effect of non-radiolabeled Tz was determined as the change in tumor uptake of [^{111}In]**16** 22 h p.i. in mice pretreated with CC49-TCO 72 h prior to Tz injection. Each non-radiolabeled Tz was administered 1 h prior to [^{111}In]**16** and the uptake normalized to a group of animals were no blocking was performed (control).

Investigated tetrazines displayed a blocking effect ranging from 69 to 99% (Table 1). No significant differences were observed for positional isomers (cf. compounds **1**-**6** and **7**-**12**). Similarly, the fluorine unsubstituted derivatives (**3**, **4**, **6**, **9**, **10** and **12**) showed a comparable blocking effect to fluorine substituted structures (**1**, **2**, **5**, **7**, **8** and **11**). Moreover, glycine analogues (**2**, **4**, **8** and **10**) displayed a similar blocking effect as their more polar analogues (**1**, **3**, **7** and **9**). In contrast, introduction of a fluoroethyl group to the nitrogen of the glycine analogues (**5**, **6**, **11** and **12**) resulted in a significant reduction of the blocking effect. These derivatives possess only a blocking effect of <80% (Table 1). In a next step, we explored the tolerability of the tetrazine towards various substituents at position 4 of the phenyl ring. Therefore, we introduced a hydroxy (**13**), methoxy (**14**) and fluoroethoxy (**15**) group in this position. All compounds also showed a blocking effect > 50.000 $M^{-1}s^{-1}$ with TCO in PBS.

Precursor Synthesis and Radiolabeling

Encouraged by these results, we decided to develop **15** into a PET tracer. The precursor (**59**) for this ligand could be synthesized over 5 synthesis steps (Scheme 2). We decided to use a nosylate leaving group since we have recently reported - for a similar scaffold - that increased RCYs up 10-fold were accessible using this leaving group compared to mesylate or tosylate leaving groups.[27] Radiolabeling of [^{18}F]**15** was carried out in a one-pot, two-step reaction sequence (Scheme 3). A protection/deprotection strategy was chosen since unprotected carboxylic acids prevent ^{18}F-fluorinations.[24] Radiolabeling was only possible using low basicity labeling conditions.[27] 3-Substituted 1,2,4,5-tetrazines are reported to be too sensitive for standard ^{18}F-fluorination approaches.[20] [^{18}F]**15** was labeled in a radiochemical yield (RCY) of 13 ± 2 % (n = 3) with a radiochemical purity (RCP) of >98% and a molar activity (A_M) of 55 ± 5 GBq/µmol. The total synthesis was approximately 90 min including separation and formulation of the final product. Maximum isolated amount was 1.1 GBq (Figure 4).

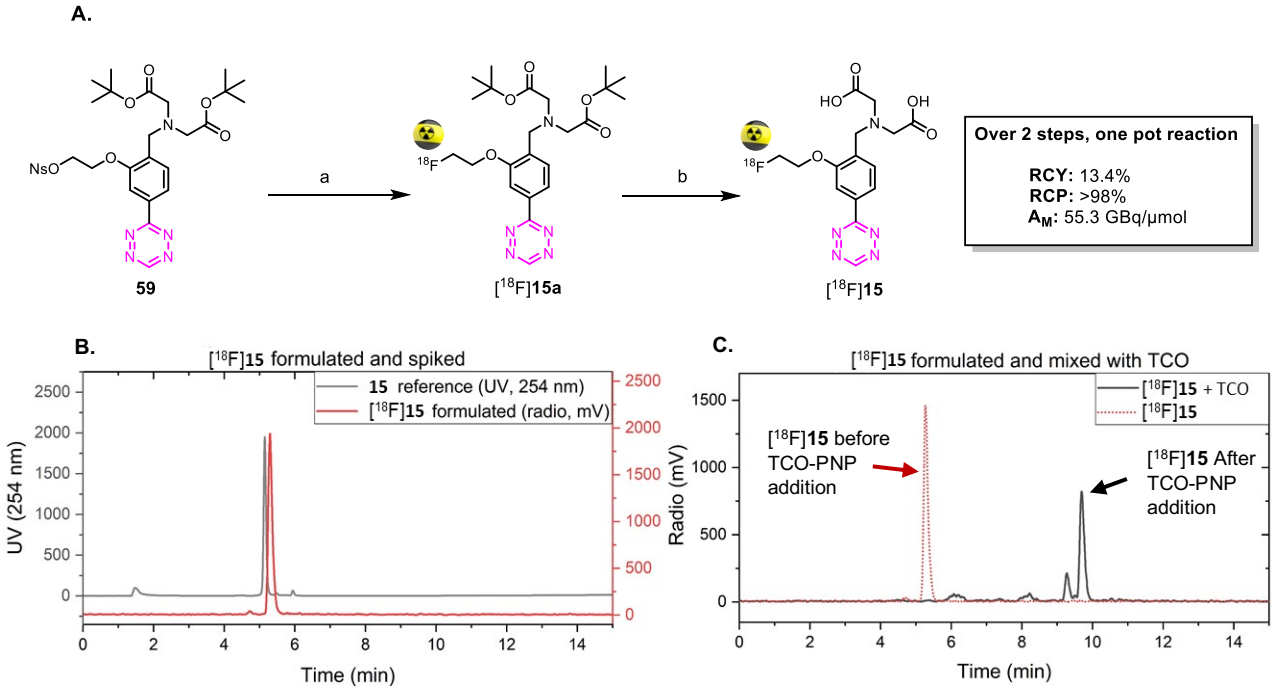


Figure 4. Radiolabeling of [^{18}F]**15**. **A**. Reaction sequence. *Reagents and conditions:* a) [^{18}F]Bu_4NF/Bu_4NOMs PO_4^{3-}, *t*-BuOH/DMSO, 100 ºC, 5 min; b) TFA, CH_3CN, 80 ºC, 10 min. **B**. Analytical chromatograms of formulated [^{18}F]**15** spiked with non-radioactive **15** for identification (rt: 5.29 min). **C**) Analytical chromatogram of the reaction between [^{18}F]**15** shows that the tetrazine core of is intact. Analytical chromatogram of [^{18}F]**15** before TCO-PNP addition (red) and after TCO-PNP addition (black).

Bench stability of [^{18}F]15 and its precursor (59)

The stability of [^{18}F]**15** and **59** was investigated by analytical HPLC. [^{18}F]**15** was stable for at least 4 hours and therefore, sufficient for most applications (Figure 5A). In contrast, **59** decomposed over time. The process could be prevented storing **59** at -20 °C as a solid (DMSO solution) - at least for 3 months (Figure 5B).

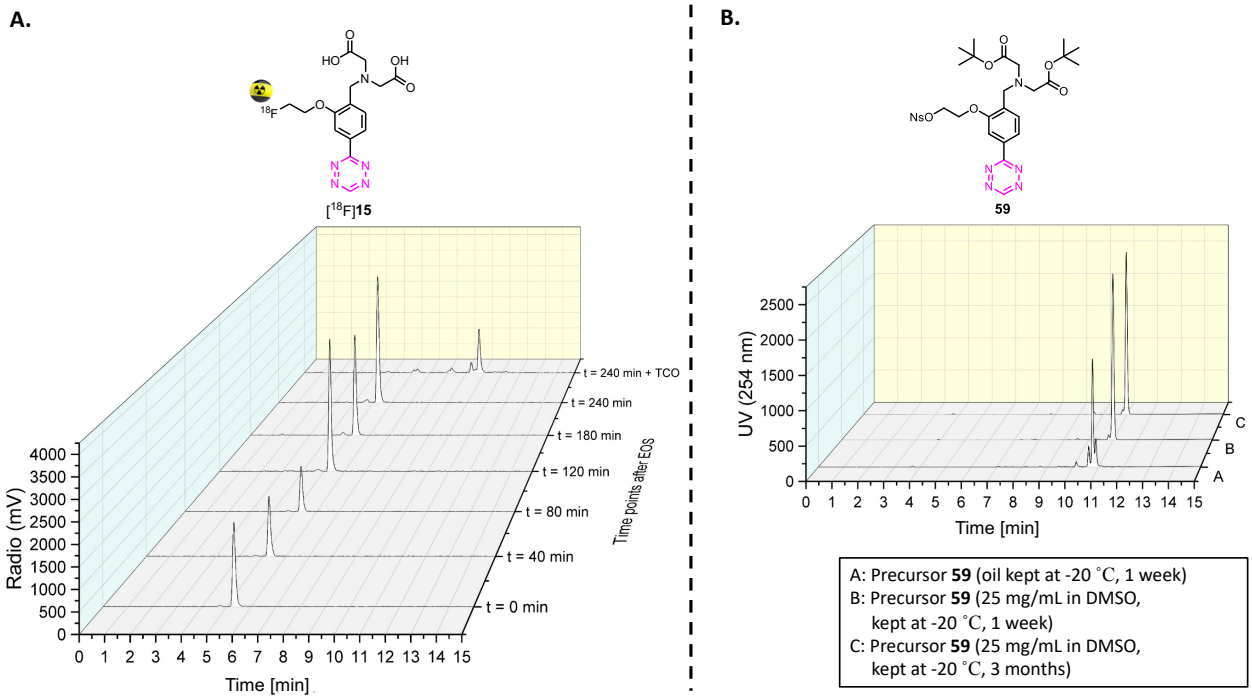
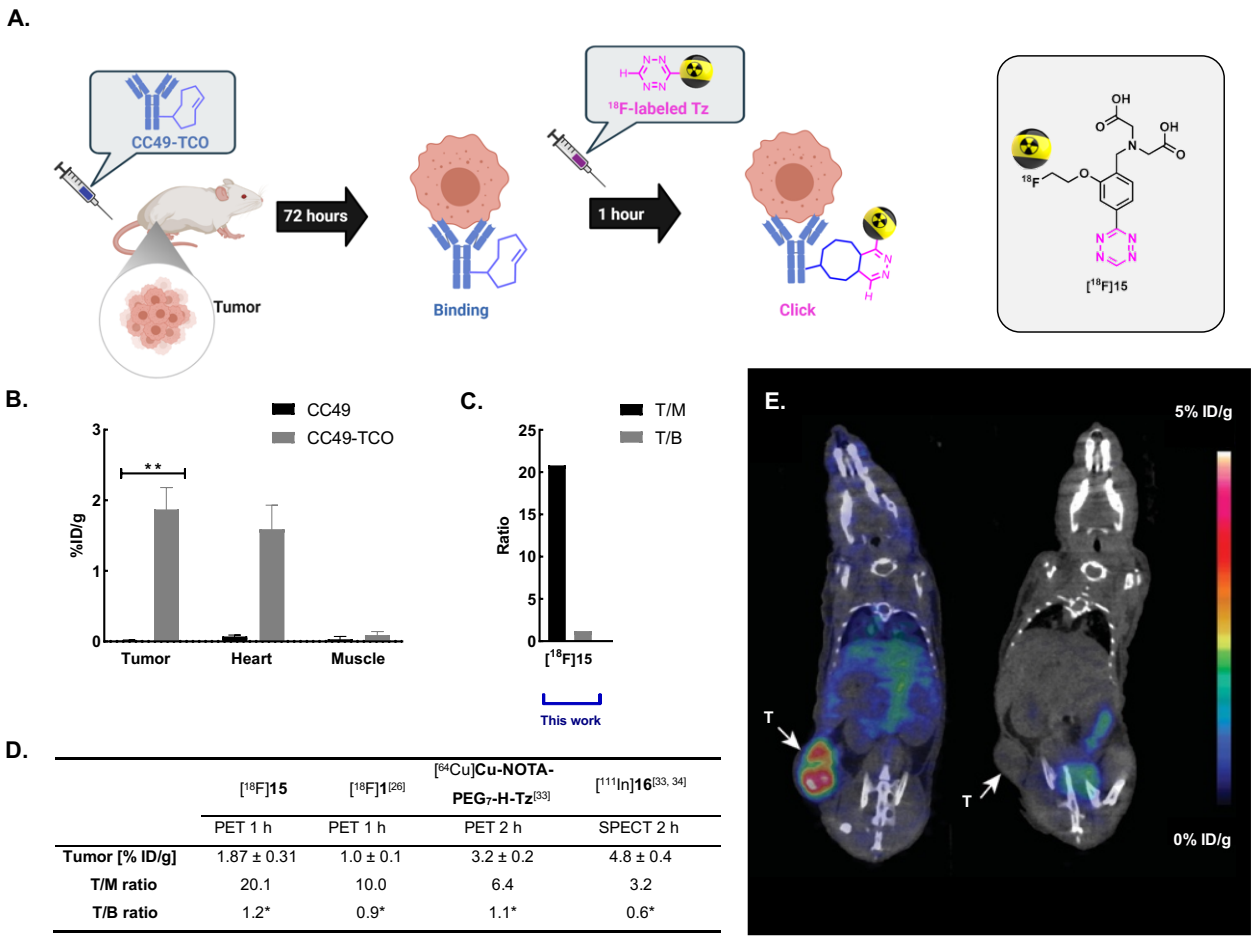


Figure 5. A. Stability of [^{18}F]**15** at r.t. over 4 hours. After 4 hours, no signs of decomposition were detected and [^{18}F]**15** was still able to react quantitatively with TCO-PNP. **B.** Stability of the precursor **59** as a pure oil at r.t., at -20 °C or dissolved (25 mg/mL) in DMSO and frozen at -20 °C. DMSO frozen samples were stable over the course of at least 3 months.

PET-imaging

The ability of [^{18}F]**15** to be used as a pretargeting imaging agent was evaluated using a similar setup that was used for the *ex vivo* blocking study. Mice were administered with CC49-TCO 72 h prior to administration of [^{18}F]**15**, where after PET/CT scanning was performed 1 h later. Control animals were injected with unconjugated mAb CC49 instead of CC49-TCO, but were otherwise treated exactly the same. After PET/CT scanning, animals were euthanized and an *ex vivo* biodistribution performed. The data are shown in Figure 6 and Table S2. PET/CT data showed that pretreated mice with CC49-TCO had a significantly higher tumor uptake (1.87 ± 0.31 %ID/g) compared to control (0.01 ± 0.01 %ID/g) (mean ± S.E.M, n = 4, p = 0.006) (Figure 6). The tumor uptake was clearly visible. A tumor-to-muscle (T/M) ratio of 20.1 and a tumor-to-blood (T/B) ratio of 1.2 from the image derived data (heart uptake used as surrogate for blood uptake) was determined. The relatively low T/B ratio, a common finding in pretargeted imaging, is likely due to ligation of [^{18}F]**15** with remnant CC49-TCO still circulating in the blood.[25, 32] Clearance of CC49-TCO from blood will most likely increase the ratio drastically.[17, 25] Uptake in all other tissues was low. *Ex vivo* biodistribution confirmed the results from the imaging experiment (Table 3S).



D.

	[^{18}F]**15**	[^{18}F]**1**[26]	[^{64}Cu]**Cu-NOTA-PEG$_7$-H-Tz**[33]	[^{111}In]**16**[33, 34]
	PET 1 h	PET 1 h	PET 2 h	SPECT 2 h
Tumor [% ID/g]	1.87 ± 0.31	1.0 ± 0.1	3.2 ± 0.2	4.8 ± 0.4
T/M ratio	20.1	10.0	6.4	3.2
T/B ratio	1.2*	0.9*	1.1*	0.6*

Figure 6: PET/CT scan of CC49-TCO pretargeted [^{18}F]**15** in LS174T tumor xenograft bearing mice. **A**. Schematic illustration of the pretargeted imaging approach. **B.** PET-Image derived mean %ID/g in tumor-, heart- and muscle tissue 1 h p.i. of [^{18}F]**15**. mean ± S.E.M, n = 4/group. *p < 0.05 (Welch's t-test) (**C.** & **D.)** Image-derived tumor uptake (mean % ID/g), Tumor-to-muscle (T/M) and Tumor-to-Blood ratio (T/B) of [^{18}F]**15** in comparison with "state-of-the-art" applied Tz imaging agents [^{18}F]**1** (PET 1 h p.i,., *n* = 3), [^{64}Cu]**Cu-NOTA-PEG$_7$-H-Tz** (PET 2 h p.i,., *n* = 4) and [^{111}In]**16** (SPECT 2 h p.i., *n* = 4). Tumor uptake and ratios of [^{18}F]**1**, [^{64}Cu]**Cu-NOTA-PEG7-H-Tz** and [^{111}In]**16** 2 h p.i. in nude BALB/c mice bearing subcutaneous LS174T tumor xenografts pretreated with CC40-TCO (100 µg) has recently been published. [26, 33, 34] Data are shown as mean ± standard error of mean (SEM). *Image-derived uptake in heart from SPECT and PET images used as a surrogate for blood.[26, 33, 34] **E.** Representative images from PET/CT-scans 1 h p.i. of [^{18}F]**15**. Mice were administered with either non-modified CC49 (left) or CC49-TCO (right), 72 h prior to [^{18}F]**15** injection. Arrows indicate LS174T tumor xenografts. Scale bar indicates mean %ID/g.

Conclusion

Pretargeted imaging of nanomedicines has the potential to revolutionize state-of-art nuclear imaging. In this study, we have developed the first aliphatic ^{18}F-Tetrazine suitable for *in vivo* pretargeted PET imaging. [^{18}F]**15** has been synthesized in sufficient yield, purity and molar activity for *in vivo* evaluation. These studies showed that [^{18}F]**15** displayed favorable pharmacokinetics and good target-to-background ratios in pretargeted experiments. [^{18}F]**15** possess the potential to be clinically translated for *in vivo* pretargeted PET imaging.

Experimental

Synthesis

All reagents and solvents were dried prior to use according to standard methods. Commercial reagents were used without further purification. Analytical TLC was performed using silica gel 60 F254 (Merck) with detection by UV absorption and/or by charring following immersion in a 7% ethanolic solution of sulfuric acid or $KMnO_4$-solution (1.5 g of $KMnO_4$, 10 g K_2CO_3, and 1.25 mL 10% NaOH in 200 mL water). Purification of compounds was carried out by column chromatography on silica gel (40-60 μm, 60 Å) or employing a CombiFlash NextGen 300+ (Teledyne ISCO). ^1H and ^{13}C NMR spectra were recorded on Brucker (400 and 600 MHz instruments), using Chloroform-*d*, Methanol-d_4 or DMSO-d_6 as deuterated solvent and with the residual solvent as the internal reference. For all NMR experiences the deuterated solvent signal was used as the internal lock. Chemical shifts are reported in δ parts per million (ppm). Coupling constants (*J* values) are given in Hertz (Hz). Multiplicities of ^1H NMR signals are reported as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; dt, doublet of triplets; t, triplet; q, quartet; m, multiplet; br, broad signal. NMR spectra of all compounds are reprocessed in MestReNova software (version 12.0.22023) from original FID's files. Mass spectra analysis was performed using MS-Acquity-A: Waters Acquity UPLC with QDa-detector. Purification by preparative HPLC was performed on Agilent 1260 infinity system, column SymmetryPrep-C18, 17 mL/min H_2O-MeCN gradient 50-100% 15 min with 0.1% trifluoroacetic acid. All final compounds were >95% pure as determined by analytical HPLC. Analytical HPLC method: (Thermo Fisher® UltiMate 3000) with a C-18 column (Luna® 5u C18(2) 100Å, 150 x 4.6 mm), eluents: A: H2O with 0.1% TFA, B: MeCN with 0.1% TFA. Gradient from 100% A -> 100% B over 15minutes, back to 100% A over 4 minutes, flow rate 1.5 mL/min. Detection by UV-absorption at λ = 254 nm on a UVD 170U detector. Compound **9** and **10** were previously reported by us but here are synthesized according to a different procedure.[25] Compound **16** was synthesized accordingly to the previously published procedures.[21]

1-Carboxy-N-(carboxymethyl)-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (1).

*Di-tert-butyl 2,2'-((3-cyano-5-fluorobenzyl)azanediyl)diacetate (**17**).*

To a solution of 3-fluoro-5-bromomethylbenzonitrile (1.09 g, 5.10 mmol) in CH_3CN (30 mL) was added K_2CO_3 (1.06 g, 7.65 mmol) and di-*tert*-butyl iminodiacetate (1.50 g, 6.12 mmol). The reaction mixture was stirred at room temperature overnight and then the solvent was concentrated under reduced pressure. The resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over $MgSO_4$, filtered and concentrated under reduced

pressure. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 1.72 g (89%) of **17** as a white solid. *Rf* = 0.24 (n-Heptane/EtOAc 90/10); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.53 (s, 1H), 7.51 – 7.42 (m, 1H), 7.23 (ddd, *J* = 7.8, 2.5, 1.4 Hz, 1H), 3.93 (s, 2H), 3.39 (s, 4H), 1.46 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.10, 162.37 (d, *J* = 250.0 Hz), 143.99 (d, *J* = 7.5 Hz), 128.08 (d, *J* = 3.1 Hz), 120.67 (d, *J* = 21.5 Hz), 117.84 (d, *J* = 24.9 Hz), 117.62 (d, *J* = 3.4 Hz), 113.56 (d, *J* = 9.6 Hz), 81.37, 56.43 (d, *J* = 1.9 Hz), 55.30, 28.12.

*Di-tert-butyl 2,2'-((3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**21**).*

The compound was obtained following the reported procedure.[28] CH_2Cl_2 (0.256 mL, 4.00 mmol), sulfur (0.257 g, 1.00 mmol, 0.25 equiv.), hydrazine monohydrate (1.6 mL, 32.00 mmol) and ethanol (4.0 mL) along with di-*tert*-butyl 2,2'-((3-cyano-5-fluorobenzyl)azanediyl)diacetate (1.55 g, 4.00 mmol) were added to a microwave vial equipped with a stir bar. The vessel was sealed, and the reaction mixture was heated to 50 °C for 24 hours, before being allowed to cool to room temperature and unsealed. Then 3 ml of CH_2Cl_2 and $NaNO_2$ (2.8 g, 40.00 mmol) in water (40 ml) were added to the now yellow mixture followed by dropwise addition of acetic acid (14 mL), producing a mixture red in color. The reaction mixture was extracted with CH_2Cl_2, washed with brine, dried with $MgSO_4$ and filtered before concentrating *in vacuo*. The crude was purified using flash chromatography (heptane/EtOAc 95/5) to yield 0.1 g (24%) of **21** as a red solid. *Rf* = 0.39 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.34 (d, *J* = 1.4 Hz, 1H), 8.14 (ddd, *J* = 9.2, 2.5, 1.5 Hz, 1H), 7.55 – 7.45 (m, 1H), 3.98 (s, 2H), 3.40 (s, 4H), 1.41 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.25, 165.78 (d, *J* = 3.2 Hz), 163.58 (d, *J* = 247.5 Hz), 157.97, 143.30 (d, *J* = 7.1 Hz), 133.46 (d, *J* = 8.7 Hz), 124.18 (d, *J* = 2.7 Hz), 120.64 (d, *J* = 21.8 Hz), 114.15 (d, *J* = 24.5 Hz), 81.34, 56.96 (d, *J* = 1.8 Hz), 55.35, 28.17.

*1-Carboxy-N-(carboxymethyl)-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**1**).*

To a solution of di-tert-butyl 2,2'-((3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.15 g, 0.36 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 hours. The solvent was then removed under reduced pressure to obtain a pink solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.08 g (51%) of **1** as a pink solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.42 (s, 1H), 8.60 (d, *J* = 1.4 Hz, 1H), 8.41 – 8.32 (m, 1H), 7.73 – 7.64 (m, 1H), 5.11 (s, 7H), 4.59 (s, 2H), 4.11 (s, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ 168.68, 165.12 (d, *J* = 3.2 Hz), 163.27 (d, *J* = 247.6 Hz), 158.24, 135.40 (d, *J* = 7.7

Hz), 135.10 (d, J = 8.6 Hz), 126.05 (d, J = 3.0 Hz), 121.71 (d, J = 22.7 Hz), 115.46 (d, J = 24.4 Hz), 57.85, 53.62; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{13}FN_5O_4]^+$: 322.09; Found: 322.13**.**

1-Carboxy-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (2)

*Tert-butyl 2-((3-cyano-5-fluorobenzyl)amino)acetate (**25**)*

To a solution of 3-(bromomethyl)-5-fluorobenzonitrile (3.34 g, 15.60 mmol) in CH_3CN (40 mL) was added K_2CO_3 (10.78 g, 78.02 mmol) and glycine tert-butyl ester hydrochloride (7.85 g, 46.81 mmol). The reaction mixture was stirred at room temperature overnight. The solvent was removed in vacuo, and the resulting mixture was diluted with water (20 mL), extracted with EtOAc (2 x 25 mL), washed with brine (30 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 3.52 g (85%) of the desired compound as a colorless oil. Rf = 0.23 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, J = 1.5 Hz, 1H), 7.35 (dt, J = 9.3, 1.8 Hz, 1H), 7.25 – 7.18 (m, 1H), 3.83 (s, 2H), 3.28 (s, 2H), 1.92 (s, 1H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.35, 162.36 (d, J = 250.1 Hz), 144.68 (d, J = 7.4 Hz), 127.53 (d, J = 3.2 Hz), 119.97 (d, J = 21.3 Hz), 117.66 (d, J = 24.8 Hz), 117.63 (d, J = 3.3 Hz), 113.66 (d, J = 9.7 Hz), 81.60, 51.96 (d, J = 1.8 Hz), 50.82, 28.12.

*Tert-butyl 2-((tert-butoxycarbonyl)(3-cyano-5-fluorobenzyl)amino)acetate (**29**)*

To a solution of *tert*-butyl 2-((3-cyano-5-fluorobenzyl)amino)acetate (1.5 g, 5.67 mmol) and Et_3N (1.90 mL, 13.62 mmol) in CH_2Cl_2 (40 mL) was added Boc_2O (1.48 g, 6.81 mmol). The reaction was stirred at room temperature for 12 h. The solution was then washed with water (50 mL) and K_2CO_3 saturated solution (50 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to afford 2.1 g of the crude. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.84 g (89%) of the desired compound as a colorless oil (60/40 unassigned rotamers mixture). Rf = 0.48 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 – 7.34 (m, 1H), 7.33 – 7.25 (m, 2H), 4.54 (s, 1.2H), 4.49 (s, 0.8H), 3.89 (s, 0.8H), 3.74 (s, 1.2H), 1.67 – 1.35 (m, 18H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 168.51, 168.43, 162.39 (d, J = 250.6 Hz), 155.62, 155.21, 143.03 (d, J = 7.3 Hz), 142.68 (d, J = 7.3 Hz), 127.11 (d, J = 3.2 Hz), 126.85 – 126.63 (m), 119.78 (d, J = 21.6 Hz), 119.25 (d, J = 21.5 Hz), 117.99 (d, J = 24.7 Hz), 117.92 (d, J = 24.8 Hz), 117.52 – 117.42 (m), 113.90 (d, J = 9.8 Hz), 81.63 (d, J = 78.3 Hz), 81.51 (d, J = 81.3 Hz), 51.17, 50.90, 49.88, 49.52, 31.87, 29.00, 28.21, 28.03, 22.67, 14.09.

*Tert-butyl 2-((tert-butoxycarbonyl)(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (**33**)*

The compound was obtained from 2-((tert-butoxycarbonyl)(3-cyano-5-fluorobenzyl)amino)acetate (1.56 g, 4.28 mmol) following the procedure reported for **21**. The resulting residue was purified using flash chromatography (n-Heptane/EtOAc 95/5) to yield 0.45 g (25%) of the desired compound as red oil (60/40 unassigned rotamers mixture). Rf = 0.41 (n-Heptane:20%EtOAC); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.27 (s, 1H), 8.37 – 8.30 (m, 1H), 8.27 – 8.20 (m, 1H), 7.40 – 7.28 (m, 1H), 4.67 (s, 1.2H), 4.60 (s, 0.8H), 3.93 (s, 0.8H), 3.79 (s, 1.2H), 1.53 – 1.41 (m, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.64, 165.56, 163.56 (d, *J* = 248.0 Hz), 163.49 (d, *J* = 248.1 Hz), 158.02, 155.73, 155.42, 142.44 (d, *J* = 6.3 Hz), 142.19 (d, *J* = 6.9 Hz), 133.73 (d, *J* = 8.9 Hz), 123.05, 122.88, 119.57 (d, *J* = 22.0 Hz), 119.08 (d, *J* = 21.7 Hz), 81.43 (d, *J* = 82.1 Hz), 81.30 (d, *J* = 91.4 Hz), 51.41, 51.06, 49.60, 49.28, 28.30, 28.26, 28.04.

*1-Carboxy-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**2**)*

To a solution of *tert*-butyl 2-((tert-butoxycarbonyl)(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)amino)-acetate (0.30 g, 0.71 mmol) in CH_2Cl_2 (5 mL) was added trifluoroacetic acid (5 mL). The reaction was stirred at room temperature for 2 hours. The solvent was then removed under reduced pressure to obtain a red solid. NMR of the crude shows full conversion. Purification by preparative HPLC afforded 0.11 g (41%) of **2** as a pink oil. ^{1}H NMR (400 MHz, CD_3OD) δ 10.32 (s, 1H), 8.52 (s, 1H), 8.30 (ddd, *J* = 9.4, 2.5, 1.5 Hz, 1H), 7.85 – 7.25 (m, 1H), 4.36 (s, 2H), 3.93 (s, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.31, 165.08 (d, *J* = 3.3 Hz), 163.31 (d, *J* = 247.8 Hz), 158.31, 135.47 (d, *J* = 8.8 Hz), 134.73 (d, *J* = 7.8 Hz), 125.19 (d, *J* = 3.1 Hz), 120.87 (d, *J* = 22.9 Hz), 115.54 (d, *J* = 24.3 Hz), 49.76 (d, *J* = 1.9 Hz), 46.57; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{11}H_{11}FN_5O_2]^+$: 264.09; Found: 264.07.

N-(3-(1,2,4,5-Tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (3)

*Di-tert-butyl 2,2'-((3-cyanobenzyl)azanediyl)diacetate (**18**)*

The compound was obtained from 3-bromomethylbenzonitrile (1.00 g, 5.10 mmol) following the procedure employed for **17**. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 1.62 g (88%) of the desired compound as a colorless oil. Rf =0.40 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.74 (t, *J* = 1.6 Hz, 1H), 7.67 (dt, *J* = 7.9, 1.5 Hz, 1H), 7.54 (dt, *J* = 7.7, 1.5 Hz, 1H), 7.42 (t, *J* = 7.7 Hz, 1H), 3.92 (s, 2H), 3.39 (s, 4H), 1.46 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.23, 140.56, 133.34, 132.33, 130.98, 129.10, 118.85, 112.40, 81.22, 56.74, 55.24, 28.15.

*Di-tert-butyl 2,2'-((3-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**22**)*

The compound was obtained from di-*tert*-butyl 2,2'-((3-cyanobenzyl)azanediyl)diacetate (1.60 g, 4.43 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 95/5) afforded 0.37 g (20%) of the desired compound as a red oil. Rf = 0.41 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.20 (s, 1H), 8.59 (s, 1H), 8.49 (d, *J* = 7.8 Hz, 1H), 7.77 (d, *J* = 7.8 Hz, 1H), 7.56 (t, *J* = 7.8 Hz, 1H), 4.02 (s, 2H), 3.45 (s, 4H), 1.46 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.38, 166.50, 157.77, 140.32, 133.92, 131.62, 129.45, 128.70, 127.27, 81.06, 57.25, 55.27, 28.16.

*N-(3-(1,2,4,5-Tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (**3**)*

The compound was obtained from di-*tert*-butyl 2,2'-((3-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)-diacetate (0.15 g, 0.36 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.08 g (53%) of **3** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.40 (s, 1H), 8.80 (t, *J* = 1.8 Hz, 1H), 8.70 (dt, *J* = 7.9, 1.4 Hz, 1H), 7.88 (dt, *J* = 7.7, 1.4 Hz, 1H), 7.77 (t, *J* = 7.8 Hz, 1H), 4.71 (s, 2H), 4.21 (s, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.63, 165.77, 158.10, 135.38, 133.11, 131.04, 130.65, 129.97, 129.29, 58.53, 53.34; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{14}N_5O_4]^+$: 304.10; Found: 304.12.

N-(3-(1,2,4,5-Tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (4)

*Tert-butyl 2-((3-cyanobenzyl)amino)acetate (**26**)*

The compound was obtained from 3-bromomethylbenzonitrile (1.80 g, 9.18 mmol) following the procedure employed for **25**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.32 g (58%) of the desired compound as a colorless oil. Rf = 0.31 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (s, 1H), 7.59 (d, *J* = 7.7 Hz, 1H), 7.54 (d, *J* = 7.7 Hz, 1H), 7.42 (t, *J* = 7.7 Hz, 1H), 3.83 (s, 2H), 3.29 (s, 2H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.41, 141.38, 132.62, 131.67, 130.80, 129.16, 118.82, 112.46, 81.44, 52.37, 50.85, 28.10.

*Tert-butyl 2-((tert-butoxycarbonyl)(3-cyanobenzyl)amino)acetate (**30**)*

The compound was obtained from *tert*-butyl 2-((3-cyanobenzyl)amino)acetate (1.16 g, 4.70 mmol) following the procedure employed for **29**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.60 g (98%) of the desired compound as a colorless oil (60/40 unassigned rotamers mixture). Rf = 0.44 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.62 – 7.36 (m, 4H), 4.53 (s, 1.2H), 4.48 (s, 0.8H), 3.85 (s, 0.8H), 3.70 (s, 1.2H), 1.50 – 1.33 (m, 18H); ^{13}C NMR (101

MHz, $CDCl_3$) δ 168.57, 155.65, 155.35, 139.76, 139.47, 132.38, 131.76, 131.23, 131.04, 130.86, 129.34, 118.67, 112.60, 81.79, 81.71, 80.91, 80.78, 51.28, 50.92, 49.54, 49.27, 28.22, 28.00, 27.38.

*Tert-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(tert-butoxycarbonyl)amino)acetate (**34**)*

The compound was obtained from *tert*-butyl 2-((*tert*-butoxycarbonyl)(3-cyanobenzyl)amino)acetate (1.45 g, 4.18 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 85/15) afforded 0.33 g (20%) of the desired compound as a red oil (60/40 unassigned rotamers mixture). Rf = 0.42 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.21 (s, 1H), 8.89 – 8.40 (m, 2H), 7.77 – 7.47 (m, 2H), 4.65 (s, 1.2H), 4.59 (s, 0.8H), 3.89 (s, 0.8H), 3.74 (s, 1.2H), 1.48 (s, 9H), 1.43 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.85, 168.81, 166.35, 157.87, 157.83, 155.80, 155.60, 132.92, 132.25, 131.91, 131.81, 129.73, 129.62, 127.53, 127.39, 127.32, 81.68, 81.58, 80.77, 80.57, 51.56, 51.15, 49.28, 49.03, 28.33, 28.29, 28.04.

*N-(3-(1,2,4,5-Tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (**4**)*

The compound was obtained from *tert*-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(*tert*-butoxy-carbonyl)amino)acetate (0.14 g, 0.36 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.08 g (62%) of **4** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.41 (s, 1H), 8.79 (s, 1H), 8.72 (d, J = 7.8 Hz, 1H), 7.85 (d, J = 7.8 Hz, 1H), 7.79 (t, J = 7.7 Hz, 1H), 4.46 (s, 2H), 4.03 (s, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.32, 165.85, 158.13, 134.02, 133.22, 132.24, 130.02, 129.26, 128.92, 50.34, 46.44; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{12}H_{11}N_5O_2]^+$: 246.09; Found: 246.11.

N-(carboxymethyl)-2-fluoro-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)ethanaminium 2,2,2-trifluoroacetate (5)

*Tert-butyl 2-((3-cyano-5-fluorobenzyl)(2-fluoroethyl)amino)acetate (**37**)*

To a solution of *tert*-butyl 2-((3-cyano-5-fluorobenzyl)amino)acetate (1.400 g, 5.23 mmol) and K_2CO_3 (1.83 g, 13.24 mmol) in CH_3CN (40 mL) was added 1-fluoro-2-iodoethane (1.38 g, 7.94 mmol). The reaction was refluxed for 24 h. The solvent was removed under reduced pressure, water (30 mL) was added, and the mixture was extracted with EtOAc (3 x 25 mL). The organic layer was dried over $MgSO_4$, filtered and evaporated in vacuo to give an oil. The residue was purified by flash column chromatography (n-Heptane/EtOAc 80/20) to afford 1.51 g (92%) of the desired compound as colorless oil. Rf = 0.30 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.41 (d, J = 2.0 Hz, 1H), 7.34 (d, J = 9.3 Hz, 1H), 7.19 – 7.12 (m, 1H), 4.51 (q, J = 4.3, 3.7 Hz, 1H), 4.39 (q, J = 4.3, 3.7 Hz, 1H), 3.87 (s, 2H), 3.27 (s, 2H), 2.99 (q, J = 4.3, 3.8 Hz, 1H), 2.95 – 2.84 (m, 1H), 1.40 (s,

9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.17, 162.41 (d, *J* = 250.2 Hz), 144.22, 127.89 (d, *J* = 3.0 Hz), 120.42 (d, *J* = 21.6 Hz), 117.85 (d, *J* = 24.9 Hz), 117.64 (d, *J* = 3.2 Hz), 113.64 (d, *J* = 9.9 Hz), 82.80 (d, *J* = 168.2 Hz), 81.50, 57.55, 55.47, 53.67 (d, *J* = 19.8 Hz), 28.16.

*Tert-butyl 2-((3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (**41**)*

The compound was obtained from *tert*-butyl 2-((3-cyano-5-fluorobenzyl)(2-fluoroethyl)-amino)acetate (1.50 g, 4.83 mmol) following the procedure employed for 2**1**. Purification by flash chromatography (n-Heptane/EtOAc 95/5) afforded 0.44 g (25%) of the desired compound as a red oil. Rf = 0.28 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 8.33 (t, *J* = 1.4 Hz, 1H), 8.18 – 8.06 (m, 1H), 7.40 (dt, *J* = 9.1, 1.9 Hz, 1H), 4.55 (t, *J* = 4.9 Hz, 1H), 4.43 (t, *J* = 4.9 Hz, 1H), 3.97 (s, 2H), 3.33 (s, 2H), 3.05 (t, *J* = 5.0 Hz, 1H), 2.98 (t, *J* = 5.0 Hz, 1H), 1.41 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.30, 165.74 (d, *J* = 3.3 Hz), 163.54 (d, *J* = 247.6 Hz), 157.98, 143.55, 133.53 (d, *J* = 8.5 Hz), 123.99 (d, *J* = 2.8 Hz), 120.33 (d, *J* = 21.9 Hz), 114.07 (d, *J* = 24.5 Hz), 82.95 (d, *J* = 168.0 Hz), 81.38, 58.04, 55.54, 53.62 (d, *J* = 20.1 Hz), 28.18.

*N-(carboxymethyl)-2-fluoro-N-(3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)ethanaminium 2,2,2-trifluoroacetate (**5**)*

The compound was obtained from *tert*-butyl 2-((3-fluoro-5-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (0.30 g, 0.86 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.26 g (71%) of **5** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.43 (s, 1H), 8.64 (d, *J* = 1.6 Hz, 1H), 8.39 (ddd, *J* = 9.3, 2.5, 1.5 Hz, 1H), 7.69 (dt, *J* = 9.0, 2.1 Hz, 1H), 5.03 – 4.94 (m, 1H), 4.90 – 4.83 (m, 1H), 4.69 (s, 2H), 4.15 (s, 2H), 3.83 – 3.71 (m, 1H), 3.71 – 3.60 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.91, 165.05 (d, *J* = 3.3 Hz), 163.29 (d, *J* = 248.0 Hz), 158.28, 135.34 (d, *J* = 8.6 Hz), 134.32 (d, *J* = 7.7 Hz), 126.16 (d, *J* = 3.1 Hz), 121.83 (d, *J* = 22.8 Hz), 115.73 (d, *J* = 24.3 Hz), 78.98 (d, *J* = 167.7 Hz), 57.82, 54.14 (d, *J* = 19.5 Hz), 52.95; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{14}F_2N_5O_2]^+$: 310.11; Found: 310.14.

N-(3-(1,2,4,5-tetrazin-3-yl)benzyl)-N-(carboxymethyl)-2-fluoroethanaminium 2,2,2-trifluoroacetate (6)

*Tert-butyl 2-((3-cyanobenzyl)(2-fluoroethyl)amino)acetate (**38**)*

The compound was obtained from *tert*-butyl 2-((3-cyanobenzyl)amino)acetate (1.50 g, 6.09 mmol) following the procedure employed for **37**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.10 g (62%) of the desired compound as a colorless oil. Rf = 0.62 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 (d, *J* = 1.7 Hz, 1H), 7.62 (d, *J* = 7.8 Hz, 1H), 7.54 (dt, *J*

= 7.8, 1.5 Hz, 1H), 7.42 (t, *J* = 7.7 Hz, 1H), 4.58 (t, *J* = 4.9 Hz, 1H), 4.46 (t, *J* = 4.9 Hz, 1H), 3.92 (s, 2H), 3.33 (s, 2H), 3.05 (t, *J* = 5.0 Hz, 1H), 2.98 (t, *J* = 5.0 Hz, 1H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.36, 140.89, 133.09, 132.12, 130.95, 129.14, 118.88, 112.46, 82.92 (d, *J* = 167.8 Hz), 81.32, 57.87, 55.50, 53.59 (d, *J* = 20.0 Hz), 28.18.

*Tert-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (**42**)*

The compound was obtained from *tert*-butyl 2-((3-cyanobenzyl)(2-fluoroethyl)amino)acetate (1.10 g, 3.76 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 95/5) afforded 0.39 g (30%) of the desired compound as a red oil. Rf = 0.37 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.19 (s, 1H), 8.55 (d, *J* = 1.7 Hz, 1H), 8.48 (dt, *J* = 7.8, 1.5 Hz, 1H), 7.68 (d, *J* = 7.6 Hz, 1H), 7.54 (t, *J* = 7.7 Hz, 1H), 4.59 (t, *J* = 5.0 Hz, 1H), 4.47 (t, *J* = 5.0 Hz, 1H), 4.01 (s, 2H), 3.37 (s, 2H), 3.09 (t, *J* = 5.0 Hz, 1H), 3.03 (t, *J* = 5.0 Hz, 1H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.44, 166.47, 157.79, 140.53, 133.67, 131.68, 129.45, 128.53, 127.26, 83.01 (d, *J* = 167.6 Hz), 81.17, 58.36, 55.52, 53.53 (d, *J* = 20.2 Hz), 28.18.

*N-(3-(1,2,4,5-tetrazin-3-yl)benzyl)-N-(carboxymethyl)-2-fluoroethanaminium 2,2,2-trifluoroacetate (**6**)*

The compound was obtained from *tert*-butyl 2-((3-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)-amino)acetate (0.30 g, 0.86 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.23 g (66%) of **6** as a red solid. ^{1}H NMR (600 MHz, CD_3OD) δ 10.39 (s, 1H), 8.81 (d, *J* = 1.8 Hz, 1H), 8.69 (dt, *J* = 8.0, 1.4 Hz, 1H), 7.89 (dt, *J* = 7.8, 1.4 Hz, 1H), 7.77 (t, *J* = 7.8 Hz, 1H), 5.07 (s, 2H), 5.02 – 4.96 (m, 1H), 4.95 – 4.85 (m, 1H), 4.73 (s, 2H), 3.83 – 3.76 (m, 1H), 3.76 – 3.70 (m, 1H); ^{13}C NMR (151 MHz, CD_3OD) δ 167.41, 165.74, 158.11 (d, *J* = 2.1 Hz), 135.28, 133.21, 130.90, 130.49, 130.08, 129.30, 78.67 (d, *J* = 167.9 Hz), 58.38, 54.00 (d, *J* = 19.4 Hz), 52.71; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{15}FN_5O_2]^+$: 292.12; Found: 292.13.

1-Carboxy-N-(carboxymethyl)-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (7)

*Di-tert-butyl 2,2'-((4-cyano-2-fluorobenzyl)azanediyl)diacetate (**19**)*

The compound was obtained from 4-(bromomethyl)-3-fluorobenzonitrile (0.85 g, 3.97 mmol) following the procedure employed for **17**. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 1.5 g (90%) of the desired compound as a colorless oil. Rf = 0.34 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (t, *J* = 7.5 Hz, 1H), 7.47 (dd, *J* = 7.9, 1.6 Hz, 1H), 7.33 (dd, *J* = 9.2, 1.7 Hz, 1H), 4.03 (s, 2H), 3.45 (s, 4H), 1.48 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ

170.21, 160.53 (d, J = 250.0 Hz), 132.26 (d, J = 14.0 Hz), 132.19 (d, J = 5.1 Hz), 128.20 (d, J = 3.8 Hz), 118.79 (d, J = 25.5 Hz), 117.73 (d, J = 2.8 Hz), 112.16 (d, J = 9.5 Hz), 81.34, 55.61, 50.06, 28.13.

*Di-tert-butyl 2,2'-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**23**)*

The compound was obtained from Di-*tert*-butyl 2,2'-((4-cyano-2-fluorobenzyl)azanediyl)diacetate (1.5 g, 3.96 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 85/15) afforded 0.25 g (15%) of the desired compound as a red oil. Rf = 0.39 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.16 (s, 1H), 8.34 (dd, J = 8.1, 1.6 Hz, 1H), 8.21 (dd, J = 10.6, 1.7 Hz, 1H), 7.80 (t, J = 7.7 Hz, 1H), 4.01 (s, 2H), 3.42 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz, Chloroform-*d*) δ 170.33, 165.69 (d, J = 3.0 Hz), 161.70 (d, J = 247.8 Hz), 157.89, 132.38, 132.31 (d, J = 4.4 Hz), 131.48 (d, J = 14.5 Hz), 124.02 (d, J = 3.4 Hz), 114.88 (d, J = 25.1 Hz), 81.21, 55.58, 50.18 (d, J = 2.9 Hz), 28.15.

*1-Carboxy-N-(carboxymethyl)-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**7**)*

The compound was obtained from di-*tert*-butyl 2,2'-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)-azanediyl)diacetate (0.24 g, 0.55 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.02 g (8%) of **7** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.41 (s, 1H), 8.50 (dd, J = 8.0, 1.6 Hz, 1H), 8.38 (dd, J = 10.9, 1.6 Hz, 1H), 7.90 (t, J = 7.7 Hz, 1H), 4.49 (s, 2H), 3.96 (s, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ 170.22, 165.29, 162.05 (d, J = 248.2 Hz), 158.17, 135.18 (d, J = 8.2 Hz), 133.50 (d, J = 3.6 Hz), 125.50 (d, J = 13.7 Hz), 123.84 (d, J = 3.5 Hz), 114.61 (d, J = 25.2 Hz), 53.85, 51.04; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{13}FN_5O_4]^+$: 322.09; Found: 322.11.

1-Carboxy-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (8)

*Tert-butyl 2-((4-cyano-2-fluorobenzyl)amino)acetate (**27**)*

The compound was obtained from 4-(bromomethyl)-3-fluorobenzonitrile (1.00 g, 4.67 mmol) following the procedure employed for **25**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 0.52 g (42%) of the desired compound as a colorless oil. Rf = 0.31 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (t, J = 7.5 Hz, 1H), 7.32 (dd, J = 7.9, 1.5 Hz, 1H), 7.21 (dd, J = 9.4, 1.6 Hz, 1H), 3.79 (s, 2H), 3.19 (s, 2H), 1.91 (s, 1H), 1.34 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.14, 160.28 (d, J = 249.5 Hz), 133.12 (d, J = 14.8 Hz), 130.95 (d, J = 5.2 Hz), 128.13

(d, *J* = 3.8 Hz), 118.69 (d, *J* = 25.5 Hz), 117.51, 112.03 (d, *J* = 9.6 Hz), 81.23, 50.85, 45.89 (d, *J* = 3.1 Hz).

*Tert-butyl 2-((tert-butoxycarbonyl)(4-cyano-2-fluorobenzyl)amino)acetate (**31**)*

The compound was obtained from *tert*-butyl 2-((4-cyano-2-fluorobenzyl)amino)acetate (1.5 g, 5.67 mmol) following the procedure employed for **29**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.87 g (90%) of the desired compound as a colorless oil (60/40 unassigned rotamers mixture). Rf = 0.34 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (t, *J* = 7.6 Hz, 0.6H), 7.53 – 7.40 (m, 1.4H), 7.39 – 7.29 (m, 1H), 4.58 (s, 1.2H), 4.54 (s, 0.8H), 3.92 (s, 0.8H), 3.80 (s, 1.2H), 1.52 – 1.41 (m, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.59, 168.54, 160.36 (d, *J* = 249.8 Hz), 160.17 (d, *J* = 250.2 Hz), 155.49, 155.24, 146.72, 131.60 (d, *J* = 5.0 Hz), 131.35, 131.06 (d, *J* = 14.9 Hz), 130.60 (d, *J* = 5.0 Hz), 128.31 (d, *J* = 3.9 Hz), 128.13 (d, *J* = 3.8 Hz), 118.96 (d, *J* = 25.3 Hz), 118.80 (d, *J* = 25.6 Hz), 117.53 (d, *J* = 3.1 Hz), 117.44 (d, *J* = 2.6 Hz), 112.55 (d, *J* = 9.5 Hz), 85.13, 81.85, 81.74, 81.09, 80.90, 50.51, 49.84, 45.88 (d, *J* = 3.9 Hz), 45.66 (d, *J* = 3.7 Hz), 28.19, 28.02, 27.98, 27.40.

*Tert-butyl 2-((tert-butoxycarbonyl)(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (**35**)*

The compound was obtained from *tert*-butyl 2-((tert-butoxycarbonyl)(4-cyano-2-fluorobenzyl)-amino)acetate (1.8 g, 4.94 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 85/15) afforded 0.49 g (24%) of the desired compound as a red oil (60/40 unassigned rotamers mixture). Rf = 0.36 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H), 8.40 (ddd, *J* = 7.9, 4.9, 1.6 Hz, 1H), 8.28 (ddd, *J* = 10.7, 7.3, 1.7 Hz, 1H), 7.64 (t, *J* = 7.7 Hz, 0.6H), 7.56 (t, *J* = 7.7 Hz, 0.4H), 4.64 (s, 1.2H), 4.60 (s, 0.8H), 3.96 (s, 0.8H), 3.83 (s, 1.2H), 1.62 – 1.36 (m, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.72, 168.68, 165.53, 165.50, 161.47 (d, *J* = 247.5 Hz), 161.28 (d, *J* = 248.1 Hz), 157.95, 157.92, 155.54, 155.40, 132.66 (d, *J* = 8.9 Hz), 132.64 (d, *J* = 8.5 Hz), 131.57 (d, *J* = 4.6 Hz), 130.74, 130.64 (d, *J* = 9.6 Hz), 130.39 (d, *J* = 15.1 Hz), 124.10 (d, *J* = 3.3 Hz), 123.90 (d, *J* = 3.4 Hz), 114.98 (d, *J* = 24.8 Hz), 114.85 (d, *J* = 24.9 Hz), 81.69, 81.59, 80.87, 80.66, 50.25, 49.68, 45.87 (d, *J* = 3.8 Hz), 45.54 (d, *J* = 3.6 Hz), 28.25, 28.22, 28.01, 27.98.

*1-Carboxy-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**8**)*

The compound was obtained from *tert*-butyl 2-((*tert*-butoxycarbonyl)(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)amino)acetate (0.10 g, 0.33 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.05 g (40%) of **8** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.44

(s, 1H), 8.54 (dd, *J* = 8.1, 1.6 Hz, 1H), 8.45 (dd, *J* = 10.8, 1.6 Hz, 1H), 7.86 (t, *J* = 7.7 Hz, 1H), 4.52 (s, 2H), 4.07 (s, 2H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.23, 165.09 (d, *J* = 2.9 Hz), 161.74 (d, *J* = 249.3 Hz), 158.27, 136.24 (d, *J* = 8.7 Hz), 133.05 (d, *J* = 3.2 Hz), 124.10, 122.61 (d, *J* = 15.4 Hz), 114.86 (d, *J* = 24.6 Hz), 43.79 (d, *J* = 3.7 Hz); HPLC-MS $[M+H]^+$ m/z calc. for $[C_{11}H_{11}FN_5O_2]^+$: 264.09; Found: 264.08.

N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (9)

*Di-tert-butyl 2,2'-((4-cyanobenzyl)azanediyl)diacetate (**20**)*

The compound was obtained from 4-(bromomethyl)-benzonitrile (0.78 g, 4.00 mmol) following the procedure employed for **17**. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 1.29 g (89%) of the desired compound as a colorless oil. Rf = 0.36 (n-Heptane/EtOAc 60/40). ^{1}H NMR (600 MHz, $CDCl_3$) *δ* 7.54 (d, *J* = 8.3 Hz, 2H), 7.49 (d, *J* = 8.2 Hz, 2H), 3.90 (s, 2H), 3.34 (s, 4H), 1.39 (s, 18H). ^{13}C NMR (151 MHz, $CDCl_3$) *δ* 170.2, 132.2, 129.5, 119.0, 111.1, 81.4, 57.2, 55.2, 28.2.

*Di-tert-butyl 2,2'-((4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**24**)*

The compound was obtained from tert-butyl di-tert-butyl 2,2'-((4-cyanobenzyl)azanediyl)diacetate (0.85 g, 2.36 mmol) following the procedure employed for **21**. Purification by flash chromatography (85/15 heptane/EtoAc) to yield **24** (0.14 g, 14%) as a red oil. Rf = 0.33 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.50 (d, *J* = 8.4 Hz, 2H), 7.59 (d, *J* = 8.4 Hz, 2H), 3.96 (s, 2H), 3.39 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz $CDCl_3$) δ 170.41, 166.44, 157.71, 144.70, 130.55, 129.84, 128.35, 81.12, 57.29, 55.29, 28.19.

*N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxy-N-(carboxymethyl)methanaminium 2,2,2-trifluoroacetate (**9**)*

The compound was obtained from di-tert-butyl 2,2'-((4-(1,2,4,5-tetrazin-3-yl)benzyl)-azanediyl)diacetate (0.13 g, 0.31 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.035 g (26%) of **9** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) *δ* 10.27 (s, 1H), 8.55 (d, *J* = 8.4 Hz, 2H), 7.68 (d, *J* = 8.4 Hz, 2H), 4.36 (s, 2H), 3.89 (s, 4H). ^{13}C NMR (101 MHz, CD_3OD) *δ* 169.51, 166.02, 158.0,2 137.53, 132.19, 131.32, 128.24, 58.01, 53.67. HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{14}N_5O_4]^+$: 304.10; Found: 304.14.

N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (10)

*Tert-butyl 2-((4-cyanobenzyl)amino)acetate (**28**)*

The compound was obtained from 4-bromomethylbenzonitrile (1.80 g, 9.18 mmol) following the procedure employed for **25**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.42 g (62%) of the desired compound as a colorless oil. Rf= 0.31 (n-Heptane/EtOAC 60/40). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.61 (d, J = 8.4 Hz, 2H), 7.46 (d, J = 8.2 Hz, 2H), 3.85 (s, 2H), 3.29 (s, 2H), 1.92 (s, 1H), 1.47 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.42, 145.34, 132.24, 128.75, 118.89, 110.94, 81.50, 52.76, 50.90, 28.12.

*Tert-butyl 2-((tert-butoxycarbonyl)(3-cyanobenzyl)amino)acetate (**32**)*

The compound was obtained from *tert*-butyl 2-((4-cyanobenzyl)amino)acetate (1.16 g, 4.70 mmol) following the procedure employed for **29**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 1.51 g (98%) of the desired compound as a colorless oil (60/40 unassigned rotamers mixture). Rf = 0.41 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.54 – 7.45 (m, 2H), 7.33 – 7.24 (m, 2H), 4.45 (s, 1.25H), 4.41 (s, 0.75H), 3.76 (s, 0.75H), 3.61 (s, 1.25H), 1.37 – 1.24 (m, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.48, 155.53, 143.78, 143.42, 132.15, 128.38, 127.86, 118.58, 111.00, 81.48, 81.43, 80.60, 80.48, 51.68, 51.26, 49.63, 49.45, 28.08, 27.87.

*Tert-butyl 2-((3-(1,2,4,5-tetrazin-4-yl)benzyl)(tert-butoxycarbonyl)amino)acetate (**36**)*

The compound was obtained from *tert*-butyl 2-((*tert*-butoxycarbonyl)(4-cyanobenzyl)amino)acetate (1.51 g, 4.36 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 85/15) afforded 0.46 g (26%) of the desired compound as a red oil (80/20 unassigned rotamers mixture). Rf = 0.36 (n-Heptane/EtOAc 80/20); ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.90 – 8.21 (m, 2H), 7.47 – 7.37 (m, 2H), 4.57 (s, 1.1H), 4.52 (s, 0.9H), 3.68 (s, 1.1H), 3.63 (s, 0.9H), 1.50 – 1.29 (m, 18H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.87, 168.80, 166.31, 166.29, 157.77, 155.89, 155.78, 143.61, 143.33, 130.72, 130.70, 128.82, 128.56, 128.55, 128.22, 81.91, 81.75, 81.08, 80.87, 51.68, 51.26, 49.46, 49.24, 28.30, 28.28, 28.05.

*N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-1-carboxymethanaminium 2,2,2-trifluoroacetate (**10**)*

The compound was obtained from *tert*-butyl tert-butyl 2-((3-(1,2,4,5-tetrazin-4-yl)benzyl)(tert-butoxycarbonyl)amino)acetate (0.2 g, 0.5 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.095 g (52%) of **10** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.24 (s, 0.6H), 10.22 (s, 0.4H), 8.52 (d, J = 8.4 Hz, 1.2H), 8.43 (d, J = 8.4 Hz, 0.8H), 7.55 (d, J = 8.1 Hz, 1.2H), 7.33 (d, J = 8.4 Hz, 0.8H), 5.49 (s, 1.2H), 4.92 (s, 0.8H), 4.89 (s, 0.8H),

4.13 (s, 1.2H); ^{13}C NMR (101 MHz, CD_3OD) δ 169.93, 167.35, 166.06, 157.95, 157.89, 139.78, 139.27, 132.23, 131.51, 129.12, 128.86, 128.25, 128.04, 55.74, 52.10, 44.96. HPLC-MS $[M+H]^+$ m/z calc. for $[C_{12}H_{11}N_5O_2]^+$: 246.09; Found: 246.10.

N-(carboxymethyl)-2-fluoro-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)ethanaminium 2,2,2-trifluoroacetate (11)

*Tert-butyl 2-((4-cyano-2-fluorobenzyl)(2-fluoroethyl)amino)acetate (**39**)*

The compound was obtained from *tert*-butyl 2-((4-cyano-2-fluorobenzyl)amino)acetate (1.30 g, 4.92 mmol) following the procedure employed for **37**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 0.51 g (33%) of the desired compound as a colorless oil (60/40 unassigned rotamer mixture). Rf = 0.35 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.68 – 7.41 (m, 2H), 7.37 – 7.31 (m, 1H), 4.71 – 4.56 (m, 3H), 4.56 – 4.47 (m, 1H), 4.46 – 4.35 (m, 1H), 4.36 – 4.25 (m, 1H), 3.95 (s, 0.8H), 3.90 (s, 1.2H), 1.44 (s, 43.6H), 1.43 (s, 5.4H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.10, 168.02, 160.43 (d, J = 249.9 Hz), 160.33 (d, J = 250.2 Hz), 156.08, 155.83, 131.82 (d, J = 4.8 Hz), 131.22 (d, J = 4.7 Hz), 130.28 (d, J = 15.0 Hz), 130.20 (d, J = 14.9 Hz), 129.50 (d, J = 5.3 Hz), 128.39, 128.35, 119.16, 119.08, 118.91, 118.83, 117.41 (d, J = 2.8 Hz), 117.36 (d, J = 2.9 Hz), 81.48 (d, J = 170.8 Hz), 65.07 (d, J = 19.8 Hz), 46.18 (d, J = 3.5 Hz), 45.63 (d, J = 3.7 Hz).

*Tert-butyl 2-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (**43**)*

The compound was obtained from *tert*-butyl 2-((4-cyano-2-fluorobenzyl)(2-fluoroethyl)-amino)acetate (0.50 g, 1.61 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 95/5) afforded 0.13 g (22%) of the desired compound as a red oil (60/40 unassigned rotamers mixture). Rf = 0.43 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.23 (s, 1H), 8.49 – 8.35 (m, 1H), 8.35 – 8.12 (m, 1H), 8.00 – 7.46 (m, 1H), 4.74 – 4.58 (m, 3H), 4.56 – 4.48 (m, 1H), 4.46 – 4.27 (m, 2H), 3.99 (s, 0.8H), 3.94 (s, 1.2H), 1.51 – 1.38 (m, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.25, 168.18, 165.48, 161.53 (d, J = 247.6 Hz), 161.42 (d, J = 247.9 Hz), 157.97, 156.13, 156.00, 133.45 – 132.72 (m), 131.79 (d, J = 4.4 Hz), 131.26 (d, J = 4.4 Hz), 129.56 (d, J = 9.4 Hz), 129.56 (d, J = 20.7 Hz), 124.19, 124.15, 82.16, 82.12, 81.57 (d, J = 170.6 Hz), 65.11 (d, J = 4.5 Hz), 64.92 (d, J = 4.6 Hz), 58.80 (d, J = 4.4 Hz), 46.11 (d, J = 3.4 Hz), 45.61 (d, J = 3.6 Hz), 28.02, 27.92.

*N-(carboxymethyl)-2-fluoro-N-(2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)ethanaminium 2,2,2-trifluoroacetate (**11**)*

The compound was obtained from *tert*-butyl 2-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoro-ethyl)amino)acetate (0.10 g, 0.43 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.05 g (43%) of **11** as a red solid. ^{1}H NMR (600 MHz, CD_3OD) δ 10.37 (s, 1H), 8.39 (ddd, *J* = 8.1, 2.9, 1.6 Hz, 1H), 8.30 – 8.24 (m, 1H), 7.67 (t, *J* = 7.7 Hz, 1H), 4.75 (s, 1H), 4.73 (s, 1H), 4.68 – 4.60 (m, 1H), 4.60 – 4.52 (m, 1H), 4.45 – 4.39 (m, 1H), 4.39 – 4.31 (m, 1H), 4.13 (s, 1H), 4.12 (s, 1H); ^{13}C NMR (151 MHz, CD_3OD) δ 171.22 (d, *J* = 5.0 Hz), 165.36 (d, *J* = 2.9 Hz), 161.39 (d, *J* = 246.5 Hz), 161.32 (d, *J* = 246.5 Hz), 158.06 (d, *J* = 3.2 Hz), 156.50, 133.61 (d, *J* = 4.4 Hz), 133.56 (d, *J* = 4.4 Hz), 130.91, 129.42 (d, *J* = 15.1 Hz), 129.25 (d, *J* = 15.1 Hz), 123.64, 114.33 (d, *J* = 3.3 Hz), 114.16 (d, *J* = 3.2 Hz), 81.31 (d, *J* = 168.7 Hz), 65.23 (d, *J* = 7.8 Hz), 65.10 (d, *J* = 8.0 Hz), 48.52 (d, *J* = 58.1 Hz), 45.72 (d, *J* = 3.8 Hz), 45.47 (d, *J* = 3.5 Hz); HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{14}F_2N_5O_2]^+$: 310.11; Found: 310.12.

N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N-(carboxymethyl)-2-fluoroethanaminium 2,2,2-trifluoroacetate (12)

*Tert-butyl 2-((4-cyanobenzyl)(2-fluoroethyl)amino)acetate (**40**)*

The compound was obtained from *tert*-butyl 2-((4-cyanobenzyl)amino)acetate (1.100 g, 4.46 mmol) following the procedure employed for **37**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 0.75 g (57%) of the desired compound as a colorless oil. Rf = 0.22 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.58 (d, *J* = 8.3 Hz, 2H), 7.48 (d, *J* = 8.3 Hz, 2H), 4.55 (t, *J* = 4.9 Hz, 1H), 4.43 (t, *J* = 4.9 Hz, 1H), 3.93 (s, 2H), 3.32 (s, 2H), 3.03 (t, *J* = 4.9 Hz, 1H), 2.96 (t, *J* = 4.9 Hz, 1H), 1.44 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.38, 145.05, 132.15, 129.18, 118.91, 110.97, 82.89 (d, *J* = 168.0 Hz), 81.25, 58.30, 55.64, 53.67 (d, *J* = 19.9 Hz), 28.16.

*Tert-butyl 2-((4-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (**44**)*

The compound was obtained from *tert*-butyl 2-((4-cyanobenzyl)(2-fluoroethyl)amino)acetate (0.73 g, 2.50 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 95/5) afforded 0.31 g (36%) of the desired compound as a red oil. Rf = 0.43 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.51 (d, *J* = 8.4 Hz, 2H), 7.56 (d, *J* = 8.1 Hz, 2H), 4.55 (t, *J* = 5.0 Hz, 1H), 4.43 (t, *J* = 5.0 Hz, 1H), 3.95 (s, 2H), 3.13 – 2.77 (m, 2H), 1.41 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.48, 166.42, 157.73, 145.06, 130.60, 129.70, 128.41, 82.98 (d, *J* = 165.8 Hz), 81.27, 58.41, 55.63, 53.70 (d, *J* = 20.0 Hz), 28.20.

*N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-N-(carboxymethyl)-2-fluoroethanaminium 2,2,2-trifluoroacetate (**12**)*

The compound was obtained from *tert*-butyl 2-((2-fluoro-4-(1,2,4,5-tetrazin-3-yl)benzyl)(2-fluoroethyl)amino)acetate (0.15 g, 0.43 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.14 g (80%) of **12** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.40 (s, 1H), 8.69 (d, *J* = 8.4 Hz, 2H), 7.85 (d, *J* = 8.4 Hz, 2H), 5.04 – 4.97 (m, 1H), 4.92 – 4.86 (m, 1H), 4.70 (s, 2H), 4.19 (s, 2H), 3.83 – 3.75 (m, 1H), 3.75 – 3.66 (m, 1H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.39, 165.81, 158.08, 134.15, 133.78, 131.94, 128.46, 78.65 (d, *J* = 167.8 Hz), 58.22 (d, *J* = 1.9 Hz), 54.06 (d, *J* = 19.4 Hz), 52.79 (d, *J* = 2.8 Hz); HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{15}FN_5O_2]^+$: 292.12; Found: 292.15.

1-Carboxy-N-(carboxymethyl)-N-(2-hydroxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (13)

*5-Cyano-2-methylphenyl acetate (**46**)*

To a solution of 3-hydroxy-4-methylbenzonitrile (3.11 g, 23.28 mmol) and Et_3N (9.74 mL, 7.07 mmol) in CH_2Cl_2 (30 mL) was added acetic anhydride (2.64 mL, 27.93 mmol). The resulting mixture was stirred at room temperature for 12 h. The organic layer was then washed with water (2 x 30 mL) and brine (2 x 30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure to give 4.05 g (99%) of compound **46** as a white solid. Rf = 0.37 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 – 7.34 (m, 1H), 7.33 – 7.03 (m, 2H), 2.45 – 2.27 (m, 3H), 2.26 – 2.16 (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.55, 149.41, 136.64, 132.04, 129.62, 125.74, 118.05, 110.64, 20.64, 16.57.

*2-(Bromomethyl)-5-cyanophenyl acetate (**47**)*

To a solution of 5-cyano-2-methylphenyl acetate (3.00 g, 17.12 mmol) and N-bromo succinimide (4.57 g, 25.68 mmol) in $CHCl_3$ (50 mL) was added AIBN (1.12 g, 6.85 mmol). The resulting solution was refluxed for 12 h. The reaction was cooled down and the organic layer was washed with water (2 x 30 mL) and brine (2 x 30 mL), dried over anhydrous Na_2SO_4, filtered and concentrated under reduced pressure. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 3.6 g (83%) of **47** as a white solid. Rf = 0.35 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 – 7.41 (m, 3H), 4.40 (s, 2H), 2.39 (s, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.16, 148.95, 135.16, 131.68, 129.83, 126.90, 117.46, 113.41, 25.86, 20.87.

*Di-tert-butyl 2,2'-((2-acetoxy-4-cyanobenzyl)azanediyl)diacetate (**48**)*

To a solution of 2-(bromomethyl)-5-cyanophenyl acetate (3.61 g, 14.17 mmol) in CH_3CN (50 mL) was added Et_3N (5.92 mL, 42.51 mmol) and di-*tert*-butyl iminodiacetate (3.65 g, 14.87 mmol). The reaction mixture was stirred at room temperature overnight and then the solvent was concentrated under reduced pressure. The resulting mixture was diluted with water (100 mL), extracted with EtOAc (3 x 40 mL), washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (90/10 Heptane/EtOAc) afforded 5.51 g (93%) of **48** as a yellow oil. Rf = 0.39 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.83 (d, *J* = 8.0 Hz, 1H), 7.52 (dd, *J* = 8.0, 1.6 Hz, 1H), 7.32 (d, *J* = 1.6 Hz, 1H), 3.90 (s, 2H), 3.37 (s, 4H), 2.33 (s, 3H), 1.45 (s, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.36, 167.03, 147.56, 135.47, 129.90, 127.91, 124.34, 116.09, 110.08, 79.34, 53.25, 49.75, 26.27, 18.77.

*Di-tert-butyl 2,2'-((4-cyano-2-hydroxybenzyl)azanediyl)diacetate (**49**)*

To a solution of di-*tert*-butyl 2,2'-((2-acetoxy-4-cyanobenzyl)azanediyl)diacetate (5.5 g, 13.14 mmol) in CH_3CN (50 mL) was added a 1M NaOH solution (20 mL). The mixture was stirred at room temperature for 12 h. The mixture then concentrated under reduced pressure and neutralized with a 1M HCl solution. The resulting slurry was extracted with EtOAc (3 x 40 mL), washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give 4.02 g (81%) of **49** as a beige solid. Rf = 0.36 (n-Heptane/EtOAc 80/20); ^{1}H NMR (600 MHz, $CDCl_3$) δ 10.05 (s, 1H), 7.14 (d, *J* = 1.5 Hz, 1H), 7.07 (dd, *J* = 7.7, 1.6 Hz, 1H), 7.04 (d, *J* = 7.7 Hz, 1H), 3.98 (s, 2H), 3.37 (s, 4H), 1.47 (s, 9H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 169.99, 157.98, 130.17, 127.17, 122.90, 120.02, 118.82, 112.86, 82.38, 55.54, 54.94, 28.11.

*Di-tert-butyl 2,2'-((2-hydroxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**52**)*

The compound was obtained from di-tert-butyl 2,2'-((4-cyano-2-hydroxybenzyl)azanediyl)diacetate (0.4 g, 1.06 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 80/20) afforded 0.11 g (24%) of the desired compound as a red solid. Rf = 0.38 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.17 (s, 1H), 9.90 (s, 1H), 8.11 (d, *J* = 1.7 Hz, 1H), 8.02 (dd, *J* = 7.8, 1.8 Hz, 1H), 7.18 (d, *J* = 7.9 Hz, 1H), 4.05 (s, 2H), 3.43 (s, 4H), 1.47 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.06, 166.43, 158.47, 157.73, 130.51, 127.12, 119.18, 116.26, 82.19, 54.98, 28.12.

*1-Carboxy-N-(carboxymethyl)-N-(2-hydroxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**13**)*

The compound was obtained from di-*tert*-butyl 2,2'-((2-hydroxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.09 g, 0.21 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.065 g (72%) of **13** as a red solid. ^{1}H NMR (400 MHz, DMSO) δ 10.57 (s, 1H), 8.89 – 7.81 (m, 2H), 7.48 (d, *J* = 7.9 Hz, 1H), 4.03 (s, 2H), 3.56 (s, 4H); ^{13}C NMR (101 MHz, DMSO) δ 172.41, 165.79, 158.58, 157.63, 132.85, 131.88, 118.99, 114.74, 54.20, 53.75; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{13}H_{14}N_5O_5]^+$: 320.10; Found: 320.12.

1-Carboxy-N-(carboxymethyl)-N-(2-methoxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (14)

*Di-tert-butyl 2,2'-((4-cyano-2-methoxybenzyl)azanediyl)diacetate (**50**)*

To a solution of compound **49** (0.4 g, 1.07 mmol) and K_2CO_3 (0.44 g, 3.19 mmol) in CH_3CN (10 mL) was added CH_3I (0.07 mL, 1.17 mmol). The reaction was refluxed for 12 h and then concentrated under reduced pressure. The resulting mixture was diluted with water (20 mL), extracted with EtOAc (3 x 20 mL), washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure to give 0.41 g (99%) of **50** a yellow oil. Rf = 0.37 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.64 (d, *J* = 7.8 Hz, 1H), 7.20 (dd, *J* = 7.7, 1.5 Hz, 1H), 7.00 (d, *J* = 1.5 Hz, 1H), 3.89 (s, 2H), 3.78 (s, 3H), 3.37 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 168.68, 155.75, 131.63, 128.74, 122.93, 117.23, 111.16, 109.44, 79.14, 53.94, 53.78, 49.49, 26.30.

*Di-tert-butyl 2,2'-((2-methoxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**53**)*

The compound was obtained from di-*tert*-butyl 2,2'-((4-cyano-2-methoxybenzyl)azanediyl)diacetate (0.41 g, 1.05 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 0.11 g (23%) of the desired compound as a red oil. Rf = 0.29 (n-Heptane/EtOAc 80/20); 1H NMR (400 MHz, CDCl3) δ 10.12 (s, 1H), 8.17 (dd, J = 7.9, 1.6 Hz, 1H), 8.02 (d, J = 1.7 Hz, 1H), 7.72 (d, J = 7.9 Hz, 1H), 3.97 (s, 2H), 3.87 (s, 3H), 3.42 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.67, 166.38, 158.41, 157.71, 133.20, 131.26, 131.09, 120.98, 109.28, 80.95, 55.79, 55.65, 51.45, 28.19.

1-Carboxy-N-(carboxymethyl)-N-(2-methoxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)methanaminium 2,2,2-trifluoroacetate (**14**)

The compound was obtained from di-*tert*-butyl 2,2'-((2-methoxy-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.09 g, 0.21 mmol) following the procedure employed for **1**.

Purification by preparative HPLC afforded 0.075 g (80%) of **14** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.41 (s, 1H), 8.29 (d, *J* = 7.4 Hz, 2H), 7.75 (d, *J* = 7.9 Hz, 1H), 4.77 (s, 2H), 4.24 (s, 4H), 4.09 (s, 3H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.07, 165.67, 159.27, 158.16, 136.00, 134.19, 121.28, 120.52, 109.97, 55.16, 53.62, 53.48; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{14}H_{16}N_5O_5]^+$: 334.11; Found: 334.13.

1-Carboxy-N-(carboxymethyl)-N-(2-(2-fluoroethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl) methanaminium 2,2,2-trifluoroacetate (15)

*Di-tert-butyl 2,2'-((4-cyano-2-(2-fluoroethoxy)benzyl)azanediyl)diacetate (**51**)*

To a solution of compound **49** (2.6 g, 6.97 mmol) and K_2CO_3 (1.92 g, 13.94 mmol) in CH_3CN (50 mL) was added 1-fluoro-2-iodoethane (1.33 g, 7.67 mmol). The reaction was refluxed for 12 h and then concentrated under reduced pressure. The resulting mixture was diluted with water (50 mL), extracted with EtOAc (3 x 50 mL), washed with brine (50 mL), dried over $MgSO_4$, filtered and concentrated under reduced pressure. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 2.71 g (92%) of the desired compound as a colorless oil. Rf = 0.25 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.73 (s, 1H), 7.30 (dd, *J* = 7.8, 1.4 Hz, 1H), 7.05 (d, *J* = 1.4 Hz, 1H), 4.91 – 4.78 (m, 1H), 4.75 – 4.63 (m, 1H), 4.34 – 4.23 (m, 1H), 4.22 – 4.16 (m, 1H), 3.98 (s, 2H), 3.44 (s, 4H), 1.45 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.51, 156.46, 134.18, 130.73, 125.48, 118.90, 114.31, 111.31, 81.46 (d, *J* = 171.9 Hz), 81.13, 67.91 (d, *J* = 20.6 Hz), 55.88, 51.69.

*Di-tert-butyl 2,2'-((2-(2-fluoroethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**54**)*

The compound was obtained from di-*tert*-butyl 2,2'-((4-cyano-2-(2-fluoroethoxy)benzyl)azanediyl)-diacetate (2.5 g, 5.91 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 90/10) afforded 0.74 g (26%) of the desired compound as a red oil. Rf = 0.25 (n-Heptane/EtOAc 80/20); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.12 (s, 1H), 8.22 (dd, *J* = 7.9, 1.6 Hz, 1H), 8.02 (d, *J* = 1.6 Hz, 1H), 7.76 (d, *J* = 7.9 Hz, 1H), 4.84 – 4.76 (m, 1H), 4.71 – 4.65 (m, 1H), 4.41 – 4.31 (m, 1H), 4.30 – 4.23 (m, 1H), 4.00 (s, 2H), 3.43 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.67, 166.24, 157.74, 157.25, 133.99, 131.10, 121.58, 110.52, 81.68 (d, *J* = 171.2 Hz), 81.00, 67.83 (d, *J* = 20.7 Hz), 55.88, 51.82, 28.17.

*1-Carboxy-N-(carboxymethyl)-N-(2-(2-fluoroethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl) methanaminium 2,2,2-trifluoroacetate (**15**)*

The compound was obtained from di-*tert*-butyl 2,2'-((2-(2-fluoroethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (0.50 g, 0.83 mmol) following the procedure employed for **1**.

Purification by preparative HPLC afforded 0.21 g (52%) of **15** as a red solid. ^{1}H NMR (400 MHz, CD_3OD) δ 10.39 (s, 1H), 8.31 – 8.22 (m, 4H), 7.76 (d, *J* = 7.8 Hz, 1H), 5.02 – 4.93 (m, 1H), 4.92 – 4.82 (m, 1H), 4.74 (s, 2H), 4.60 – 4.56 (m, 1H), 4.54 – 4.42 (m, 1H), 4.21 (s, 4H); ^{13}C NMR (101 MHz, CD_3OD) δ 167.64, 158.16 (d, *J* = 7.4 Hz), 135.64, 134.19, 122.40, 120.89, 111.00, 81.42 (d, *J* = 168.8 Hz), 68.38 (d, *J* = 19.9 Hz), 54.10, 53.62; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{15}H_{17}FN_5O_5]^+$: 366.12; Found: 366.11.

Di-tert-butyl 2,2'-((2-(2-(((4-nitrophenyl)sulfonyl)oxy)ethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl) azanediyl)diacetate (59)

*3-(2-Hydroxyethoxy)-4-methylbenzonitrile (**55**)*

3-Hydroxy-4-methylbenzonitrile (2.0 g, 10.0 mmol) was dissolved in NaOH aqueous solution (25 mL, 15.0 mmol), 2-bromoethanol (1.06 mL, 15.0 mmol) was added. The resulting mixture was heated at 90 ℃ for 12 hours. The reaction was then cooled to room temperature, diluted with water (30 mL) and extracted with CH_2Cl_2 (2 x 50 mL), The organic layer was washed with 10% NaOH (2 x 30 mL), water (30 mL) and brine (30 mL) and dried over Na_2SO_4 . The solvent was evaporated under reduced pressure to give 1.72 (65%) of the desired compound as white solid. Rf = 0.25 (n-Heptane/EtOAc 70/30); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.24 – 7.15 (m, 2H), 7.04 (d, *J* = 1.4 Hz, 1H), 4.10 (dd, *J* = 5.1, 3.8 Hz, 2H), 4.01 (dd, *J* = 5.2, 3.7 Hz, 2H), 2.29 (s, 3H), 1.95 (s, 1H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.81, 133.17, 131.38, 125.01, 119.05, 113.82, 110.30, 69.70, 61.30, 16.65.

*4-(Bromomethyl)-3-(2-hydroxyethoxy)benzonitrile (**56**)*

To a solution of 3-(2-hydroxyethoxy)-4-methylbenzonitrile (1.30 g, 7.33 mmol) and N-bromosuccinimide (1.43 g, 8.07 mmol) in $CHCl_3$ (40 mL) was added AIBN (0.48 g, 2.93 mmol). The reaction was refluxed for 24 h. The solvent was removed under vacuum and the crude purified by flash chromatography (n-Heptane/EtOAc 80/20) to give 1.10 g (58%) of the desired compound as a white solid. Rf = 0.25 (n-Heptane/EtOAc 70/30); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.42 (d, *J* = 7.8 Hz, 1H), 7.30 – 7.22 (m, 1H), 7.13 (d, *J* = 1.4 Hz, 1H), 4.53 (s, 2H), 4.21 (dd, *J* = 4.9, 3.8 Hz, 2H), 4.04 (d, *J* = 4.5 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 156.70, 131.64, 131.33, 125.11, 118.24, 114.93, 113.64, 70.36, 61.13.

*Di-tert-butyl 2,2'-((4-cyano-2-(2-hydroxyethoxy)benzyl)azanediyl)diacetate (**57**)*

The compound was obtained from 4-(bromomethyl)-3-(2-hydroxyethoxy)benzonitrile (0.90 g, 3.51 mmol) following the procedure employed for **17**. Purification by flash chromatography (n-Heptane/EtOAc 60/40) afforded 0.90 g (61%) of the desired compound as a colorless oil. Rf = 0.24

(n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.35 (d, *J* = 7.7 Hz, 1H), 7.16 (dd, *J* = 7.7, 1.4 Hz, 1H), 7.04 (d, *J* = 1.5 Hz, 1H), 4.08 (dd, *J* = 4.8, 3.2 Hz, 2H), 3.91 (s, 2H), 3.87 (t, *J* = 4.7 Hz, 2H), 3.32 (s, 4H), 1.38 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.47, 157.89, 132.74, 132.00, 124.71, 118.70, 115.34, 112.32, 81.39, 71.02, 60.86, 55.66, 52.48, 28.12.

*Di-tert-butyl 2,2'-((2-(2-hydroxyethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)diacetate (**58**)*

The compound was obtained from di-*tert*-butyl 2,2'-((4-cyano-2-(2-hydroxyethoxy)benzyl)-azanediyl)diacetate (0.9 g, 2.14 mmol) following the procedure employed for **21**. Purification by flash chromatography (n-Heptane/EtOAc 70/30) afforded 0.26 g (25%) of the desired compound as a red oil (obtained with a 20% of inseparable impurity). Rf = 0.21 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.13 (s, 1H), 8.14 (dd, *J* = 7.9, 1.6 Hz, 1H), 8.07 (d, *J* = 1.6 Hz, 1H), 7.44 (d, *J* = 7.8 Hz, 1H), 4.24 (dd, *J* = 4.9, 3.2 Hz, 2H), 4.00 (s, 2H), 3.93 – 3.87 (m, 2H), 3.39 (s, 4H), 2.29 (s, 1H), 1.39 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.54, 166.17, 158.69, 157.78, 132.53, 132.50, 132.24, 120.90, 111.86, 81.31, 71.13, 61.09, 55.60, 52.65, 28.16.

*Di-tert-butyl 2,2'-((2-(2-(((4-nitrophenyl)sulfonyl)oxy)ethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl) azanediyl)diacetate (**59**)*

To a solution of di-*tert*-butyl 2,2'-((2-(2-hydroxyethoxy)-4-(1,2,4,5-tetrazin-3-yl)benzyl)azanediyl)-diacetate (0.09 g, 0.19 mmol) and DIPEA (0.13 mL, 0.75 mmol) in CH_2Cl_2 (20 mL) was added Nosyl chloride (0.12 g, 0.57 mmol) and DMAP (0.005 g, 0.04 mmol). The reaction was stirred at room temperature for 4 hours. The solvent was then evaporated under reduced pressure. Purification by flash chromatography (n-Heptane/EtOAc75/25) afforded 0.075 g (60%) of **59** as a red oil. Rf = 0.31 (n-Heptane/EtOAc 60/40); ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.20 (s, 1H), 8.41 (d, *J* = 8.8 Hz, 2H), 8.27 (dd, *J* = 8.0, 1.5 Hz, 1H), 8.18 (d, *J* = 8.8 Hz, 2H), 7.96 (d, *J* = 1.5 Hz, 1H), 7.80 (d, *J* = 8.0 Hz, 1H), 4.56 (dd, *J* = 5.7, 3.4 Hz, 2H), 4.36 (dd, *J* = 5.7, 3.4 Hz, 2H), 3.97 (s, 2H), 3.47 (s, 4H), 1.46 (s, 18H); ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.56, 166.04, 157.79, 156.63, 150.85, 141.68, 133.96, 131.25, 131.05, 129.32, 124.61, 121.94, 110.49, 81.10, 69.02, 65.97, 55.74, 51.88, 28.18; HPLC-MS $[M+H]^+$ m/z calc. for $[C_{29}H_{37}N_6O_{10}S]^+$: 661.23; Found: 661.24.

Reaction Kinetics Measurement

Reaction kinetics of tetrazines with standard TCO were determined using pseudo-first-order measurements with an excess of the TCO compound in PBS (pH = 7.4) at 37.0 ± 0.1 °C following the decrease of tetrazine absorbance at 535 nm. Measurements were performed in triplicates using an SX20 stopped-flow photometer (Applied Photophysics, UK) equipped with a 535 nm LED light

source. Data analysis was performed using Prism 6 (GraphPad) to determine the observed rate constants which were converted into second order rate constants through dividing by the TCO concentration. Concentrations, observed rate constants and calculated second-order rate constants are shown in Table 1.

Blocking assay and *ex vivo* studies

Establishing tumor xenografts in mice

All animal studies were approved by the Danish Animal Welfare Council, ministry of Justice. Five weeks old female nude BALB/c mice (Charles River, Sulzfeld, Germany) were allowed to acclimatize for one week. At all time the animals had access to water and chow ad libitum. Human colon cancer cell line, LS174T (ATCC, VA, USA) was cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% *L*-glutamine, 1% sodium pyruvate, 1% non-essential amino acids, and 1% penicillin-streptomycin. At a confluence of 70–90%, the cells were harvested by trypsinization and subcutaneous tumors were established in the flank of the animals by inoculation of ~ 5×10^6 LS174T cells (in 100 µL sterile PBS). The tumors were allowed to grow for 7–10 days and were measured using a caliper. The tumor volume was estimated using the formula: volume = ½ (length x width^2).

Blocking experiments

Tumor-bearing animals were matched in groups based on their tumor volume (tumor volumes of ~ 100–300 mm^3, n = 3 in each group) and were administered 100 µg/100 µL of CC49-TCO (~7 TCO/mAb) per mouse. After 3 days, animals were first injected with non-radioactive Tz (39 nmol) and after 1 h they were administered with [^{111}In]**16** (3-5 MBq/100 µL, 3.9 nmol) via the tail vein. Tz [^{111}In]**16** was radiolabeled as previously described.[9] 22 h later the mice were euthanized, and tumor, blood, heart, lung, liver, spleen, kidney, and muscle tissue were resected, weighted, and the radioactivity measured using a gamma counter (Wizard2, Perkin Elmer). Data was corrected for decay, tissue weight and injected amount of radioactivity. Tumor uptake of [^{111}In]**16** in the animals receiving the non-radiolabeled Tz was normalized to a control group of animals receiving [^{111}In]**16** exclusively. The precursor of [^{111}In]**16** was included as a positive control.

Radiochemistry

[^{18}F]Fluoride production and general methods

[^{18}F]Fluoride was produced by a cyclotron CTI Siemens Eclipse, Rigshospitalet, Denmark, by irradiating [^{18}O]H_2O via a (p,n) reaction. Automated synthesis was performed on a Scanys synthesis

module (Scansys Laboratorieteknik, Denmark) and analytical HPLC was performed on a Thermo Fisher UltiMate 3000 equipped with a C18 column (Luna 5 µm C18(2) 100 Å, 150 mm × 4.6 mm). Eluents: A, H_2O with 0.1% TFA; B, MeCN with 0.1% TFA. Gradient from 100% A to 100% B over 15 min, back to 100% A over 4 min, flow rate 1.5 mL/min. Detection by UV absorption at λ = 254 nm on a UVD 170U detector and radioactivity was analyzed with a flow-through GM tube based radiodetector (Scansys).

Radiolabeling

The aqueous [^{18}F]fluoride solution received from the cyclotron was passed through a preconditioned anion exchange resin (Sep-Pak Light QMA cartridge). The QMA was preconditioned by flushing it with 10 mL 0.5 M K_3PO_4 and washing it with 10 mL H_2O afterwards. [^{18}F]F^- was eluted from the QMA into a 4 mL v-shaped vial with 1 mL Bu_4NOMs dissolved in MeOH. The eluate was dried at 100 °C for 5 min under N_2-flow. **59** was dissolved in 167 µL DMSO and then diluted with 833 µL tBuOH. The solution was added to the dried fluoride solution an allowed to react for 5 min at 100 °C. The reaction was cooled to 50 °C with air before addition of 3 mL H_2O. This mixture was applied to a Sep-pak plus C18 solid phase extraction (SPE) cartridge that was preconditioned by flushing it with 10 mL EtOH followed by 10 mL of H_2O. The SPE was flushed with another 5 mL of H_2O and dried with N_2. The product was eluted from the SPE with 2 mL MeCN into a 7 mL v-shaped vial containing 600 µL TFA. This mixture was reacted for 10 min at 80 °C. The reaction was then concentrated under N_2-flow for 20 min to reduce the solvent volume to <0.1 mL. To this crude product mixture, 2.5 mL of H_2O was added, and this solution purified by semipreparative HPLC (Luna 5 µm C18(2) 100 Å, 250 mm × 10 mm, isocratic, 70% EtOH in H_2O with 0.1% TFA 3 mL/min (rt: 13 min). The product was collected in a 20 mL vial and diluted with 100 mM sterile phosphate buffer to adjust the pH to 5-8. The max EtOH concentration was 5% and the activity concentration was 30-80 MBq/mL.

Tetrazine core reactivity test

The reaction between [^{18}F]**15** TCO-PNP was performed by mixing the formulated [^{18}F]**15** (200 µL) with 5 µL of the commercially available TCO-PNP ester dissolved in DMF (5 mg/mL) in an analytical HPLC vial. The solution was gently shaken and left for 1 min before it was injected on the analytical HPLC for analysis.

Pretargeted Imaging

Pretargeted imaging of [^{18}F]**15** as tested *in vivo* using the TAG-72 targeting antibody CC49 in human colorectal cancer xenograft tumors LS174T. Tumors were established in 7-8 week old Balb/c nude

mice and after one week, the animals were injected i.v. with either 50 µg TCO-modified CC49 (CC49-TCO, ~7 TCO/mAb, 2 nmol TCO/mouse), or non-modified CC49 (control) (n = 4 per group). 72 hours later, ^{18}F-UB201 (1.74 ± 0.319 (mean ± SD) MBq/100 µL (55.3 GBq/µmol) were injected i.v., followed by PET/CT-scan (Inveon®, Siemens Medical Solutions), one-hour post injection. PET data was acquired in an energy window of 350–650 keV and a time resolution of 6 ns followed by a CT scan (360 projections, 65 kV, 500 µA and 400 ms). PET and CT images were aligned by rigid affine registration, after which 3D regions of interest (ROI) were created on the full CT tumor volume, as well as heart and muscle tissue to quantify uptake of [^{18}F]**15** (Figure 6 and Table S2). Additionally, selected organs were harvested for *ex vivo* biodistribution. The radioactivity in the tissues were determined using a gamma counter (Wizard^2, Perkin Elmer). Data was corrected for decay, tissue weight and the injected dose of radioactivity. GraphPad Prism 9 (GraphPad Software) was used for statistical analyses and plotting data. Statistical difference between mean %ID/g values were analyzed using Welch's T-test. Results were considered significant when $p < 0.05$.

Author Contributions

[†]UMB and KB contributed equally to the work. Organic synthesis was done by UMB and subsequent radiolabeling experiments was performed by KB. *In vivo* studies and PET experiments were performed by JJ, LH and VS. HM evaluated the reaction kinetics of all the compounds in the manuscripts. The study was conceptionally designed by all the authors and the manuscript written by UMB, KB and MMH with contribution from all authors. All authors have given approval to the final version of the manuscript.

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Development of the First Aliphatic ^{18}F-Labeled Tetrazine Suitable for Pretargeted PET Imaging – Expanding the Bioorthogonal Tool Box. Umberto M. Battisti, Klas Bratteby, Jesper T. Jørgensen, Lars Hvass, Vladimir Shalgunov, Hannes Mikula, Andreas Kjaer, and Matthias M. Herth