

Article

Development of ^{18}F -Labeled Bispyridyl Tetrazines for In Vivo Pretargeted PET Imaging

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Abstract: Pretargeted PET imaging is an emerging and fast-developing method to monitor immunoncology strategies. Currently, tetrazine ligation is considered the most promising bioorthogonal reaction for pretargeting in vivo. Recently, we have developed a method to ^{18}F -label ultrareactive tetrazines by copper-mediated fluorinations. However, bispyridyl tetrazines—one of the most promising structures for in vivo pretargeted applications—were inaccessible using this strategy. We believed that our successful efforts to ^{18}F -label H-tetrazines using low basic labeling conditions could also be used to label bispyridyl tetrazines via aliphatic nucleophilic substitution. Here, we report the first direct ^{18}F -labeling of bispyridyl tetrazines, their optimization for in vivo use, as well as their successful application in pretargeted PET imaging. This strategy resulted in the design of [^{18}F]45, which could be labeled in a satisfactorily radiochemical yield (RCY = 16%), molar activity ($A_m = 57 \text{ GBq}/\mu\text{mol}$), and high radiochemical purity (RCP > 98%). The [^{18}F]45 displayed a target-to-background ratio comparable to previously successfully applied tracers for pretargeted imaging. This study showed that bispyridyl tetrazines can be developed into pretargeted imaging agents. These structures allow an easy chemical modification of ^{18}F -labeled tetrazines, paving the road toward highly functionalized pretargeting tools. Moreover, bispyridyl tetrazines led to near-instant drug release of iTCO-tetrazine-based ‘click-to-release’ reactions. Consequently, ^{18}F -labeled bispyridyl tetrazines bear the possibility to quantify such release in vivo in the future.

Keywords: bioorthogonal chemistry; tetrazine ligation; bispyridyl tetrazines; pretargeted imaging; PET; fluorine-18; molecular imaging



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1. Introduction

Radioimmunoconjugates have emerged as important tools; e.g., in the diagnosis and treatment of cancer [1,2]. A subclass within these important substances are monoclonal antibodies (mAbs). They can be designed and produced with exquisite target affinity and selectivity. From a nuclear molecular imaging point of view, mAb-based agents can result in high target-to-background ratios with a low nondisplaceable binding component, which make them almost ideal tracers. Unfortunately, at the same time they possess slow pharmacokinetic properties; i.e., target accumulation and blood clearance takes days rather than hours [3,4]. Consequently, long-lived radionuclides must be used to match the pharmacokinetic profile of these vectors [3]. This results in unnecessary radiation burden for

patients (Figure 1A), and sometimes even at a magnitude prohibitive for clinical studies [5]. An elegant way to overcome these limitations is a relatively new technique based on a pretargeted approach. In this approach, the accumulation timeframe of mAbs is temporally decoupled from the actual imaging process. In particular, a tagged mAb is administered days before a small molecule imaging agent is used to bioorthogonally react with the tag of the mAb. In this way, the accumulation of the mAb can be imaged even after days with the aid of a short-lived radionuclide (Figure 1B) [6].

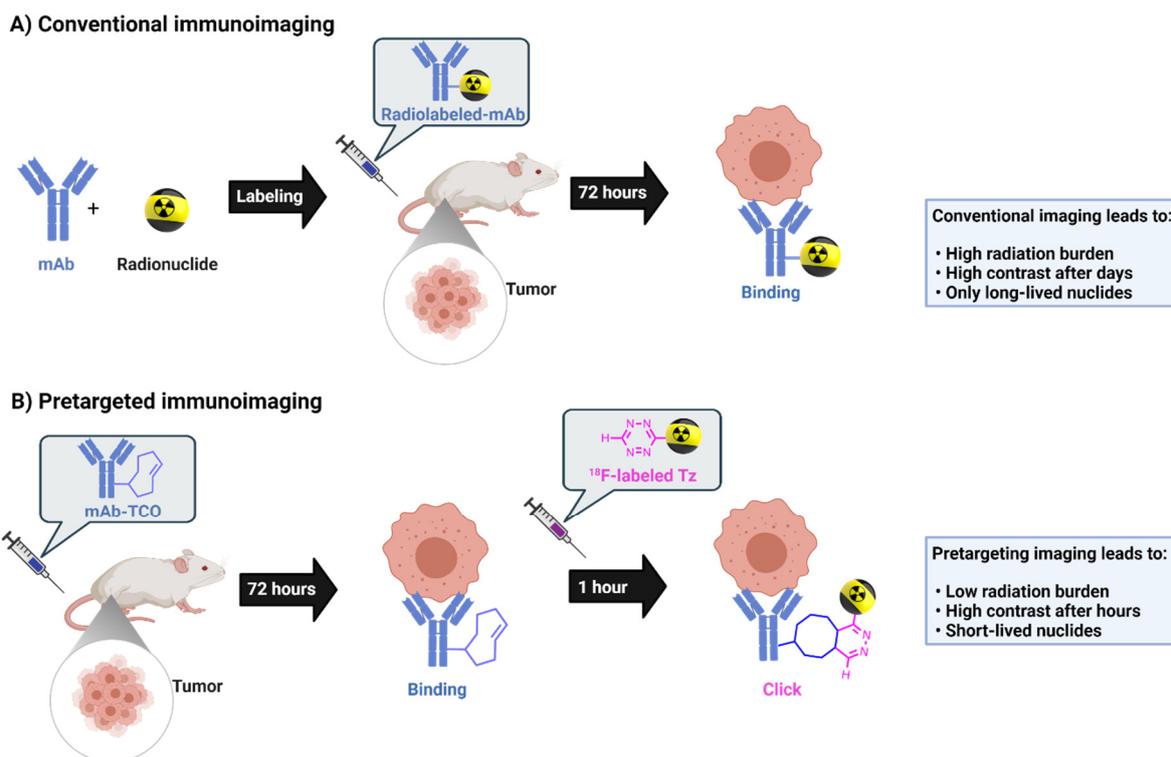


Figure 1. (A) Conventional immunoimaging vs. (B) pretargeted immunoimaging based on tetrazine ligation [3].

Pretargeted imaging consequently exploits the unique targeting properties of mAbs and the rapid pharmacokinetics properties of small molecules, enabling exceptional target-to-background ratios within hours [3]. Currently, the most promising reaction for pretargeted imaging is the tetrazine ligation between a tetrazine (Tz) and a trans-cyclooctene (TCO) (Figure 1B). Several preclinical examples have been reported that demonstrated the potential of this reaction [6–22]. More importantly, in late 2020, the first clinical phase I trial based on tetrazine ligation was initiated (NCT04106492) [23]. From a clinical point of view, a fluorine-18 (^{18}F)-labeled Tz would be ideal for positron emission tomography (PET) applications, since ^{18}F possesses almost perfect physical characteristics for molecular imaging [24–26]. However, due to the intrinsic instability of the tetrazine scaffold to basic conditions—typically employed in direct ^{18}F -fluorination approaches—no ^{18}F -tetrazine was synthesized until few years ago [10,27–31]. Recently, we reported the first direct ^{18}F -fluorination of highly reactive tetrazines, which could be labeled via copper-mediated oxidative ^{18}F -fluorination [32]. This methodology allowed us to develop a low-lipophilicity, fast-clearing, and highly reactive tetrazine PET tracer (1) (Figure 2). In a similar way, we were able to develop the first direct aliphatic ^{18}F -radiolabeled Tz (2) (Figure 2). This was possible using ultralow basic conditions [33,34]. Both compounds (1 and 2) showed high imaging contrast during in vivo experiments, and are currently subjects in further studies to evaluate their potential to be translated into the clinic.

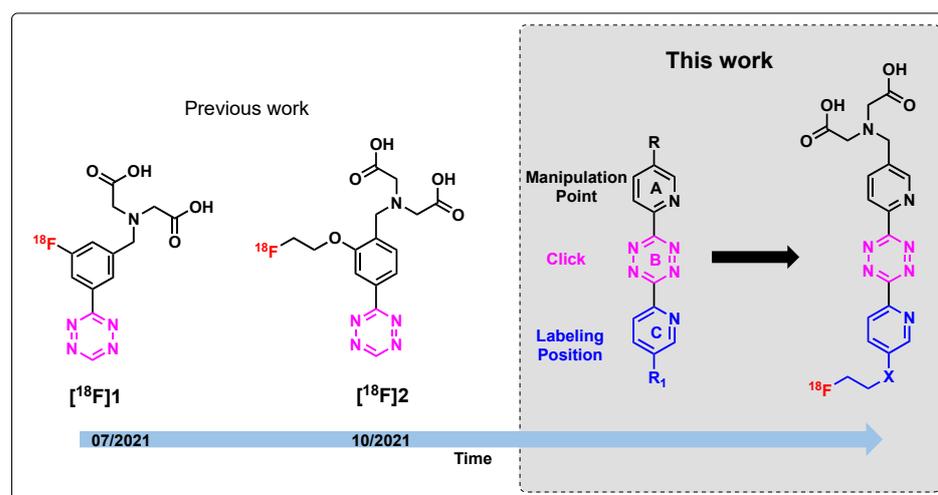


Figure 2. Compound evaluation and rationale—from previous work to this project: The ^{18}F -labeling of H-Tz has already been established (previous work); labeling of bispyridyl Tzs succeeded in this study. Bispyridyl Tzs have intrinsic high-reaction kinetics and allow higher chemical flexibility. Pyridyl unit A can be used to introduce the necessary polarity for in vivo pretargeting, whereas pyridyl unit B can be used for labeling purposes.

However, both labeling methods (Cu-mediated ^{18}F -fluorination and direct aliphatic ^{18}F -labeling) have thus far only been successful for H-Tzs. Bispyridyl scaffolds could not be labeled with Cu-mediated approaches due to the chelating effect of the pyridyl nitrogen [32,35]. Aliphatic labeling was only applied on monosubstituted Tzs [34]. Bispyridyl Tzs are, however, very appealing subjects to be used for pretargeted experiments (Figure 2). Beside H-Tzs, these structures are the only other structural class that have been successfully applied in vivo [6,35–38]. Bispyridyl Tzs have intrinsic high-reaction kinetics and allow for greater flexibility with respect to structural modifications compared to H-Tzs. For example, one pyridyl unit can be used for labeling purposes, whereas the other can be used to introduce polarity or other functional scaffolds as NIR turn-on dyes or additional chelators for pretargeted radiotherapy [3,17,39,40]. Recently, bispyridyl Tzs also were shown to be superior for instant and near-quantitative ‘click-to-release’ approaches [41]. Radiolabeled bispyridyl Tzs could be used as such to quantify drug release in vivo.

In this work, we describe the design, synthesis, and in vivo evaluation of the first ^{18}F -labeled bispyridyl Tz. In order to develop such a Tz, we exploited one pyridyl ring to introduce polarity. We recently showed that a $\text{clogD}_{7.4}$ of at least -3 is a necessary parameter for successful in vivo pretargeting [17]. The other pyridyl ring was used for labeling purposes using a set of different linkers. The selection of the linker for the ^{18}F -labeling was initially performed using H-Tzs due to their more accessible chemistry. Promising structures in term of radiochemical conversions (RCC) were translated into their bispyridyl Tz counterparts. Finally, the best candidate was applied for pretargeted PET experiments.

2. Results and Discussion

2.1. Evaluation of Different Fluoroethyl Linkers Using H-Tz as Model Structures

We have previously reported that fluoroethyl moieties can be used to introduce ^{18}F -fluoride into H-Tz employing non-nucleophilic bases [33,34]. We believed that these conditions could also be suitable to label bispyridyl-based Tzs. In order to test the influence on the labeling step of different conjugation strategies between the fluoroethyl moiety and the Tz, we synthesized a set of structures possessing various linkers such as esters, amines, ethers and amides (Figure 3). We decided to use H-Tzs for this study, as they are easier to access. Seven different molecules (3–9) and their corresponding precursors were designed.

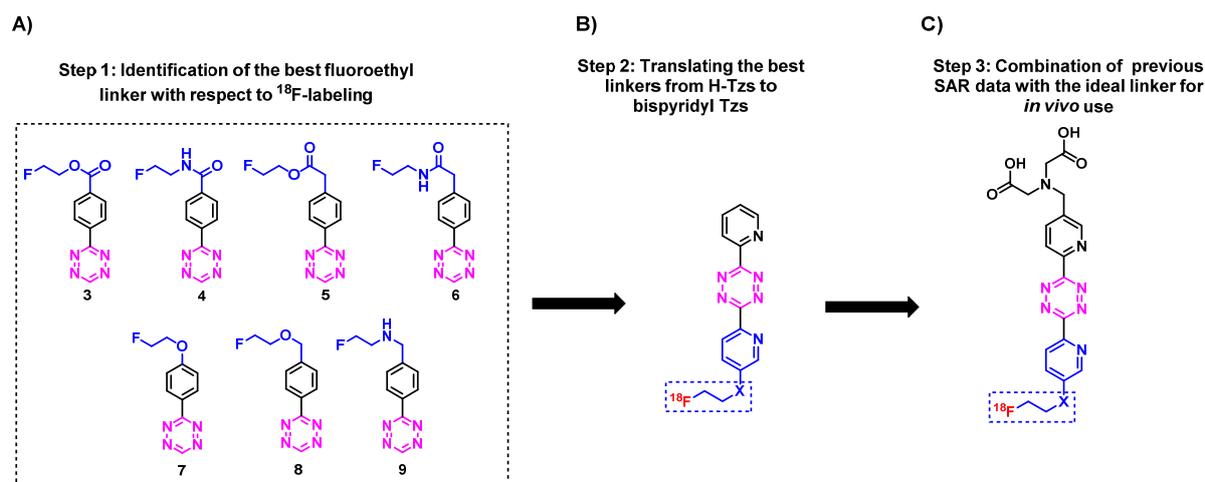
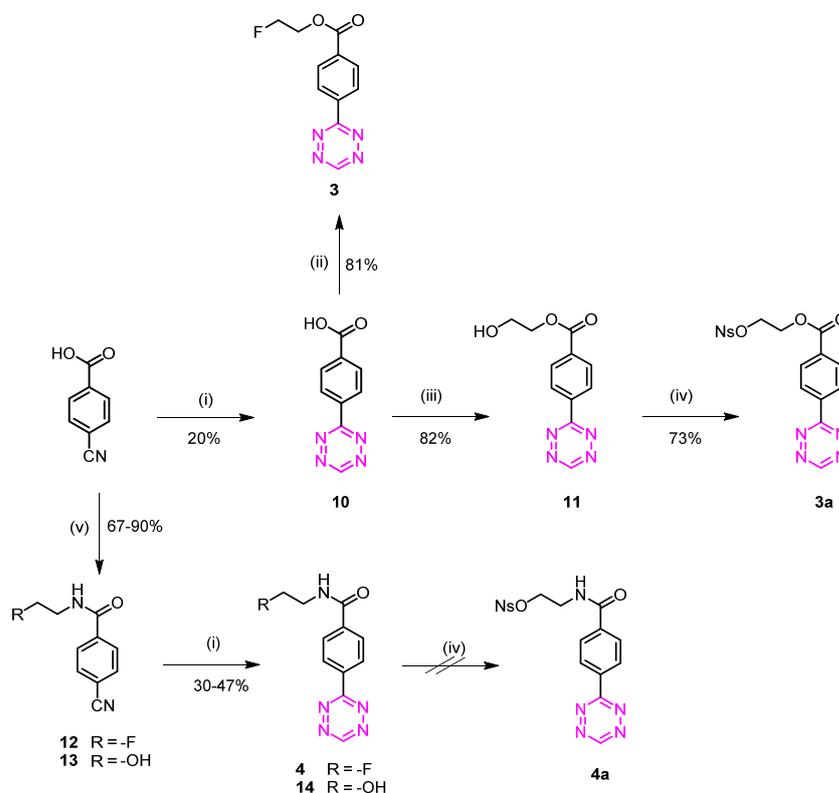


Figure 3. Compound design strategy. (A) Screening of fluoroethyl linkers. H-Tzs were initially selected to evaluate different types of linkers due to their easier chemical accessibility. (B) The best linkers in terms of RCC were translated on the bispyridyl Tz scaffold. (C) The most promising structure was developed into a potential PET tracer [17].

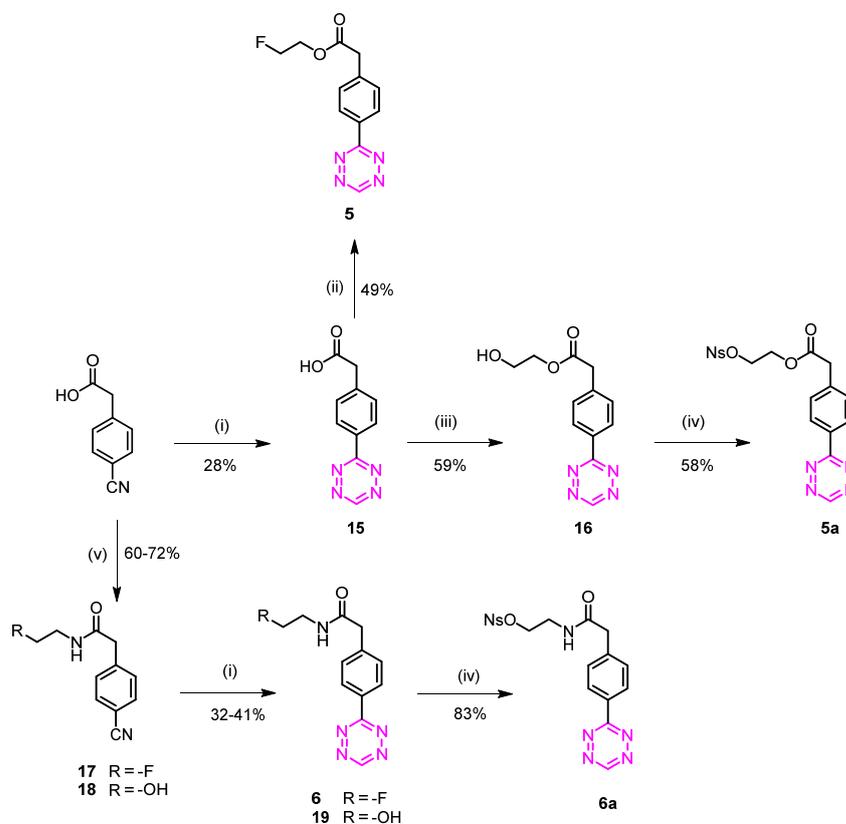
2.2. Synthesis of H-Tz Derivatives

Compound **8** and its precursor **8a** were synthesized as previously reported [33]. Compounds **3** and **4** and their corresponding precursor were obtained as reported in Scheme 1.



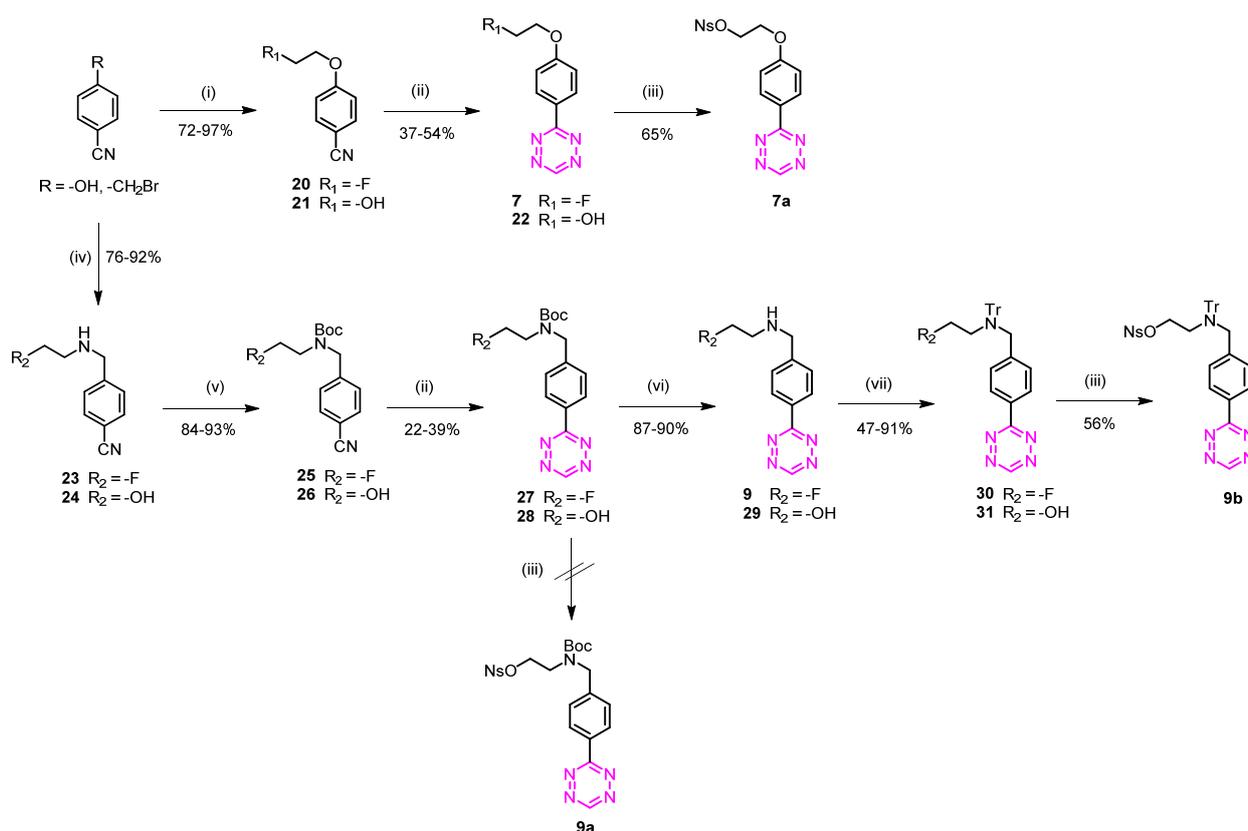
Scheme 1. Reagents and conditions: (i) (a) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, CH_2Cl_2 , S_8 , EtOH, 50°C , 24 h; (b) NaNO_2 , AcOH, 0°C to rt, 30 min; (ii) 1-fluoro-2-iodoethane, DIPEA, DMF, 70°C , 4 h; (iii) 2-bromoethanol, DIPEA, DMF, 70°C , 4 h; (iv) nosyl chloride, DIPEA, DMAP, CH_2Cl_2 , rt, 1 h; (v) 2-fluoroethylamine hydrochloride or ethanolamine, Et_3N , CDI, CH_3CN , 0°C to rt, 12 h.

Briefly, Tz **10** was synthesized from 4-cyanobenzoic acid using a Pinner-like, sulfur-mediated procedure in modest yields [42]. Subsequent alkylation with 1-fluoro-2-iodoethane or 2-bromoethanol respectively gave compounds **3** and **11**. The latter was then reacted with nosyl chloride to yield precursor **3a**. Coupling of 4-cyanobenzoic acid with 2-fluoroethylamine or ethanolamine afforded **12** and **13**; these were then converted into the corresponding Tz derivatives **4** and **14**. These intermediates were further nosylated under basic conditions. However, we were not able to isolate **4a**, since an intramolecular substitution occurred that could not be prevented. Tzs **5** and **6** and their respective precursors were obtained in a similar manner as reported in Scheme 2. The yields obtained were comparable to those of **3** and **4**. However, the amide precursor **6a** could be isolated in a yield of 83%. This most likely was possible due the lower reactivity of the tetrazine core compared to that of **4a**.



Scheme 2. Reagents and conditions: (i) (a) $\text{NH}_2\text{NH}_2 \cdot \text{H}_2\text{O}$, CH_2Cl_2 , S_8 , EtOH, 50°C , 24 h; (b) NaNO_2 , AcOH, 0°C to rt, 30 min; (ii) 1-fluoro-2-iodoethane, DIPEA, DMF, 70°C , 4 h; (iii) 2-bromoethanol, DIPEA, DMF, 70°C , 4 h; (iv) nosyl chloride, DIPEA, DMAP, CH_2Cl_2 , rt, 1 h; (v) 2-fluoroethylamine or ethanolamine, CDI, CH_3CN , 0°C to rt, 12 h.

Compounds **7** and **9** were obtained using a different synthesis strategy (Scheme 3). 4-Hydroxybenzonitrile was reacted with 1-fluoro-2-iodoethane or 2-bromoethanol to respectively give **20** and **21** in 71% and 92% yields. These were converted into the corresponding Tzs **7** and **22** in modest yields (37% and 54%, respectively). The latter was further modified to give the nosylate precursor **7a**. Intermediates **17** and **18** were obtained from 4-(bromomethyl)benzonitrile and the corresponding amines. N-Boc protection and reaction with hydrazine gave Tzs **27** and **28**. Deprotection of **27** afforded compound **9** in an almost quantitative yield. Reaction of nosyl chloride with **28** resulted in an intramolecular reaction, and **9a** could not be obtained. For this reason, a different protective group was selected. Then, **28** was deprotected and reacted with trityl chloride to give **31**. In order to have the corresponding reference compound, **9** was converted to **30** as well. Finally, reaction with nosyl chloride gave **9b** in a yield of 56%.



Scheme 3. Reagents and conditions: (i) 1-fluoro-2-iodoethane or 2-bromoethanol, K_2CO_3 , CH_3CN , $70\text{ }^\circ\text{C}$, 12 h; (ii) (a) $NH_2NH_2 \cdot H_2O$, CH_2Cl_2 , S_8 , $EtOH$, $50\text{ }^\circ\text{C}$, 24 h; (b) $NaNO_2$, $AcOH$, $0\text{ }^\circ\text{C}$ to rt, 30 min; (iii) nosyl chloride, DIPEA, DMAP, CH_2Cl_2 , rt, 1 h; (iv) 2-fluoroethylamine or ethanolamine, CH_3CN , $0\text{ }^\circ\text{C}$ to rt, 12 h; (v) Boc_2O , Et_3N , CH_2Cl_2 , $0\text{ }^\circ\text{C}$ to rt, 12 h; (vi) HCl , dioxane, rt, 4 h; (vii) $TrtCl$, Et_3N , CH_2Cl_2 , $0\text{ }^\circ\text{C}$ to rt, 12 h.

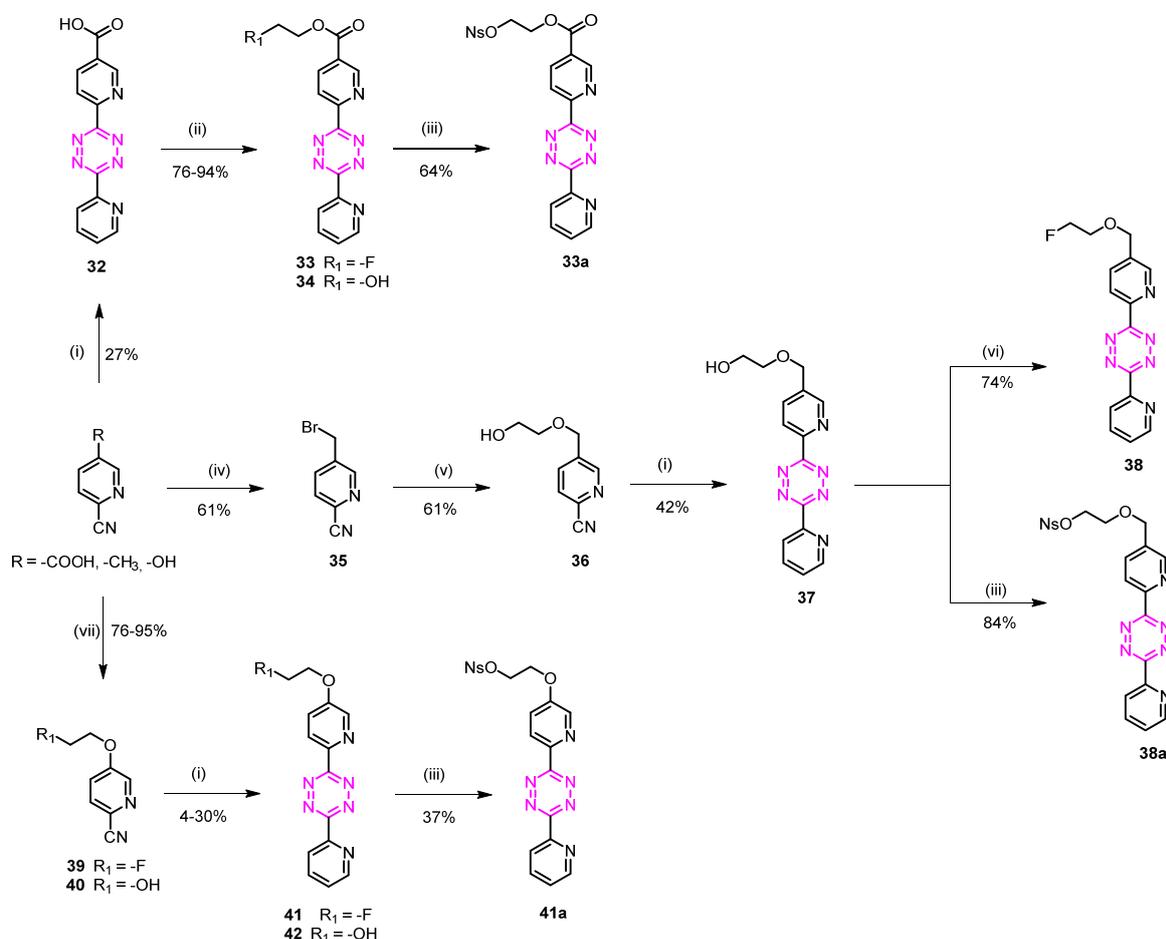
2.3. Labeling of H-Tz Derivatives

As we recently reported, nosyl leaving groups are optimally suited to aliphatically ^{18}F -label high-reactive Tzs. A mesylate or tosylate base precursor resulted in low radiochemical yields (RCY) [33,43]. Radiolabeling only succeeded using low basicity conditions, since H-Tzs are too sensitive for standard—rather, basic—aliphatic ^{18}F -fluorination approaches [29,33]. Within this study, we applied the same labeling conditions. Furthermore, we also investigated to what extent the precursor concentration influenced the RCC. Two amounts (3.1 and 9.3 nmol) were selected in this respect. With the exception of compound 3, both concentrations resulted in the same RCC range. Labeling attempts revealed further that the linkers influenced RCCs strongly. Only tetrazines 3, 7, and 8 could be synthesized. RCCs were in the range of 23–53% (based on radio-HPLC, $n = 3$). No or only minimal product formation could be observed with a different substitution profile. In light of that, bispyridyl Tzs 33, 38, and 41 were designed, synthesized, and then radiolabeled.

2.4. Synthesis of the Bispyridyl Tzs

Bispyridyl analogues of 3, 7, and 8 were synthesized as described in Scheme 4. 6-Cyanonicotinic acid was converted to the corresponding bispyridyl Tz 32 following a previously reported procedure [44]. The latter was alkylated with 1-fluoro-2-iodoethane or 2-bromoethanol to respectively give compounds 33 and 34. The alcohol derivative was then transformed to the nosylate precursor 33a. Differently, 5-methylpicolinonitrile was brominated and reacted with ethylene glycol to afford 36. Formation of the Tz ring and conversion to the fluorine analogue gave 38 [45]. Nosylation of the same alcohol

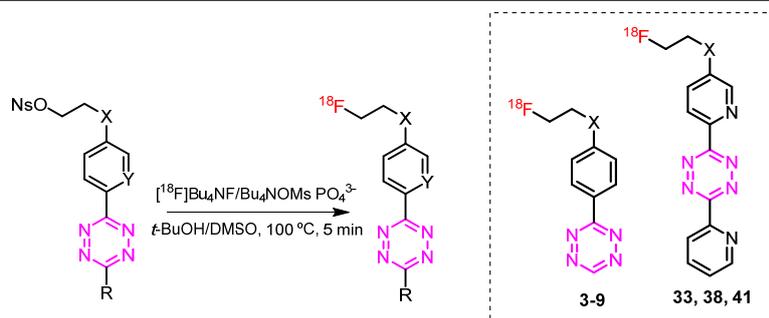
intermediate yielded **38a**. The last compounds, **41** and **41a**, were obtained starting with 5-hydroxypicolinonitrile and using the same procedure employed for Tz **7**.



Scheme 4. Reagents and conditions: (i) 2-pyridinecarbonitrile, (a) $NH_2NH_2 \cdot H_2O$, S_8 , EtOH, $90^\circ C$, 4 h; (b) $NaNO_2$, AcOH, $0^\circ C$ to rt, 30 min; (ii) 1-fluoro-2-iodoethane or 2-bromoethanol, DIPEA, DMF, $70^\circ C$, 4 h; (iii) nosyl chloride, DIPEA, DMAP, CH_2Cl_2 , rt, 1 h; (iv) NBS, AIBN, CH_3CN , reflux, 12 h; (v) ethylene glycol, NaH, THF, $0^\circ C$ to reflux, 12 h; (vi) $CF_3(CF_2)_3SO_2F$, $Et_3N \cdot 3HF$, THF, rt, 18 h; (vii) 1-fluoro-2-iodoethane or 2-bromoethanol, K_2CO_3 , CH_3CN , $70^\circ C$, 12 h.

2.5. Labeling of Bispyridyl Tz Derivatives

Compounds $[^{18}F]33$, $[^{18}F]38$, and $[^{18}F]41$ were labeled from their corresponding precursors **33a**, **38a**, and **41a** following the same procedure reported in Section 2.3. The results are reported in Table 1. As expected from previous results, radiolabeling was successful in all cases. RCCs were in the range of 20–30%. However, only compounds $[^{18}F]33$ and $[^{18}F]38$ could be radiolabeled in a similar conversion when lower precursor concentrations were used. Due to that and its easier synthetic access compared to **33**, we decided to use **38** as a template for our next steps, aiming to develop a pretargeted imaging agent.

Table 1. ^{18}F -labeling of tetrazines developed in this study.

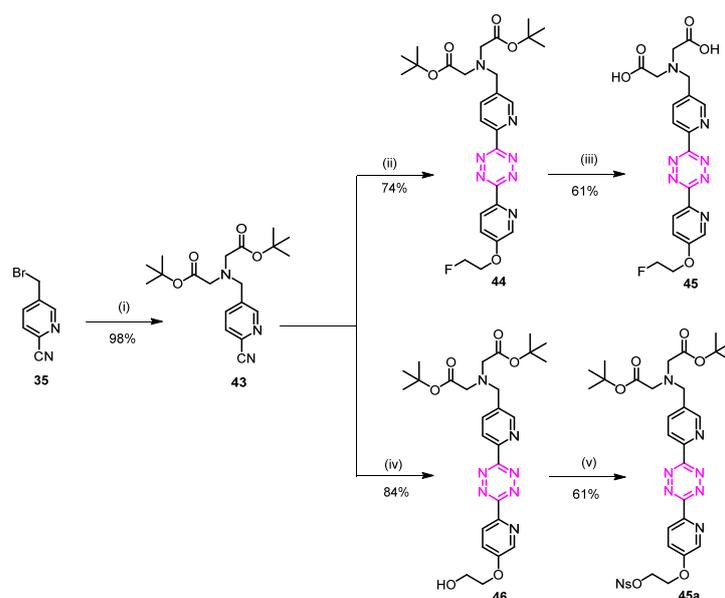
Tetrazine	X	R	RCC (%) *		RCC (%) *	
			Low Precursor Amount (3.1 nmol)		High Precursor Amount (9.3 nmol)	
			HPLC	TLC	HPLC	TLC
3	-COO-	-H	0	0	18 ± 8	16 ± 6
5	-CH ₂ COO-	-H	_ ^b	_ ^b	_ ^b	_ ^b
6	-CH ₂ CONH-	-H	_ ^b	_ ^b	_ ^b	_ ^b
7	-O-	-H	45 ± 6	52 ± 4	53 ± 5	55 ± 4
8	-CH ₂ O-	-H	23 ± 4	24 ± 3	26 ± 4	28 ± 3
9 ^c	-CH ₂ NH-	-H	_ ^d	_ ^d	_ ^d	_ ^d
33	-COO-	-2-Pyr	24 ± 3	22 ± 3	27 ± 4	25 ± 5
38	-O-	-2-Pyr	20 ± 11	21 ± 8	29 ± 10	28 ± 6
41	-CH ₂ O-	-2-Pyr	9 ± 1	8 ± 1	23 ± 3	26 ± 6

^a Precursor was not obtained. ^b No reaction observed. ^c Precursor protected with trityl. ^d Precursor decompose during reaction. n.d. = not determined. * Radiochemical conversions (RCC) were calculated for each compound by radio-HPLC and radio-TLCs as recently reported [43]. All results were based on $n = 3$ experiments.

2.6. Synthesis and Evaluation of 45 by Ex Vivo Blocking Assay

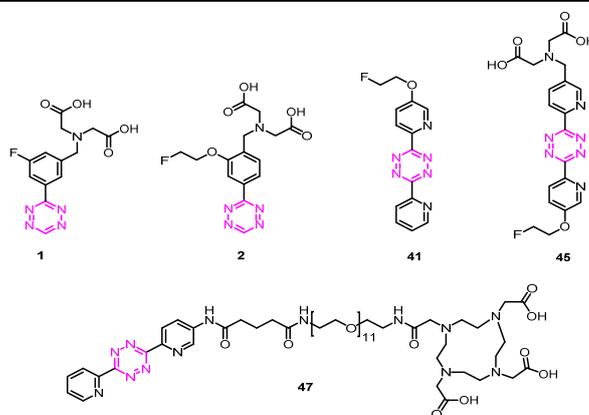
Compound **45** was designed based on a fluorothexy moiety identified to yield in the highest RCCs of previous results in this study. To decrease lipophilicity, we further conjugated a diacetic acid moiety to the structure. This combination should have resulted in an easy-to-label compound, as well as an imaging agent with the necessary polarity to be applied for in vivo pretargeted imaging. The pathway to synthesize **45** is depicted in Scheme 5. Alkylation of di-*tert*-butyl iminodiacetate by compound **35** under basic conditions yielded **43**. The latter was reacted with an excess of **39** and hydrazine hydrate to give **44**. Deprotection and purification by preparative-HPLC afforded **45** as a TFA salt.

Next, **45** was evaluated for its abilities in pretargeting. We recently developed a blocking assay that allowed us to assess the in vivo ligation performance of unlabeled tetrazine derivatives, omitting the time-consuming development of radiolabeled tetrazines for each tested ligand. It was based on the ability of Tzs to block the binding of the literature-known pretargeted imaging agent [^{111}In]**47** to the pretargeting vector CC49-TCO (administered 72 h prior) in tumor-bearing mice [17,32,34]. In this setup, **45** showed almost complete blocking. This blocking effect was as good as the ones observed with currently successfully applied ‘state-of-the-art’ pretargeted imaging agents (Table 2). This result allowed us to assume that **45** could indeed be a suitable candidate for pretargeted imaging.



Scheme 5. Synthesis of **45** and its precursor. Reagents and conditions: (i) di-*tert*-butyl iminodiacetate, K_2CO_3 , CH_3CN , reflux, 12 h; (ii) **39**, (a) $NH_2NH_2 \cdot H_2O$, S_8 , EtOH, $90^\circ C$, 4 h; (b) $NaNO_2$, AcOH, $0^\circ C$ to rt, 30 min; (iii) TFA, CH_2Cl_2 , rt, 4 h; (iv) **40**, (a) $NH_2NH_2 \cdot H_2O$, S_8 , EtOH, $90^\circ C$, 4 h; (b) $NaNO_2$, AcOH, $0^\circ C$ to rt, 30 min; (v) nosyl chloride, DIPEA, DMAP, CH_2Cl_2 , rt, 1 h.

Table 2. Structural scaffolds, calculated physicochemical properties (TPSA, $clogD_{7.4}$), and blocking efficiencies of all investigated tetrazine derivatives. Compound **45** displayed a suitable profile to be translated into preclinical studies.



Tetrazine	$clogD_{7.4}$ ^b	TPSA ^c	Blocking Effect ^f	% Tumor Uptake of [¹¹¹ In] 47 after Blocking
1 ^a	−6.93	129	90 ± 5 ^g	10 ± 5 ^g
2 ^a	−6.83	138	98 ± 0.3 ^h	2 ± 0.3 ^h
41 [*]	3.65	35	22 ± 21	78 ± 21
45 ^a	−6.36	164	99 ± 0.7	1 ± 0.7
47 ^d	−4.13 ^e	358	99 ± 0.5	1 ± 0.5

^a The compounds were obtained as trifluoroacetate salt. ^b Calculated distribution coefficient at physiological pH (7.4) using Chemicalize software. ^c Calculated using Chemicalize software. ^d The compound was employed as a reference. ^e Calculated as chelated to a trivalent cation. ^f The blocking effect of nonradiolabeled Tz was determined as the change in tumor uptake of [¹¹¹In]**47** 22 h p.i. Each Tz was administered 1 h prior to [¹¹¹In]**47**, 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 Materials and Methods section. ^g Blocking data from [26]. ^h Blocking data from [27]. ^{*} Tz **41** was tested to demonstrate that the blocking effect was related to $clogD_{7.4}$ and not the bispyridyl scaffold. TPSA = topological polar surface area.

2.7. Synthesis and Labeling of [^{18}F]45

The precursor (**45a**) was synthesized over 4 synthesis steps (Scheme 5). Briefly, **40** was reacted with an excess of **43** and hydrazine hydrate to give, after oxidation, **46**. This was then converted to the corresponding nosylated derivative **45a**. Radiolabeling of [^{18}F]45 was carried out in a one-pot, two-step reaction sequence (Figure 4A,B). The conditions were similar to those we recently reported and applied for all former labeling attempts in this study [29,32,33,46]. A protection/deprotection strategy was needed to successfully label [^{18}F]45 in a radiochemical yield (RCY) of $16 \pm 4\%$ ($n = 4$), a radiochemical purity (RCP) of $>98\%$, and a molar activity (A_M) of $57 \pm 15 \text{ GBq}/\mu\text{mol}$. The total synthesis was approximately 90 min, including labeling, purification, and formulation of the final product. The maximum isolated amount was 1.2–2 GBq (EOS) starting from $\sim 17 \text{ GBq}$ of fluoride-18. The [^{18}F]45 rapidly reacted with TCO-PNP carbonate, and was stable in PBS at room temperature for minimum of 4 h, as confirmed by radio-HPLC (Figure 4C).

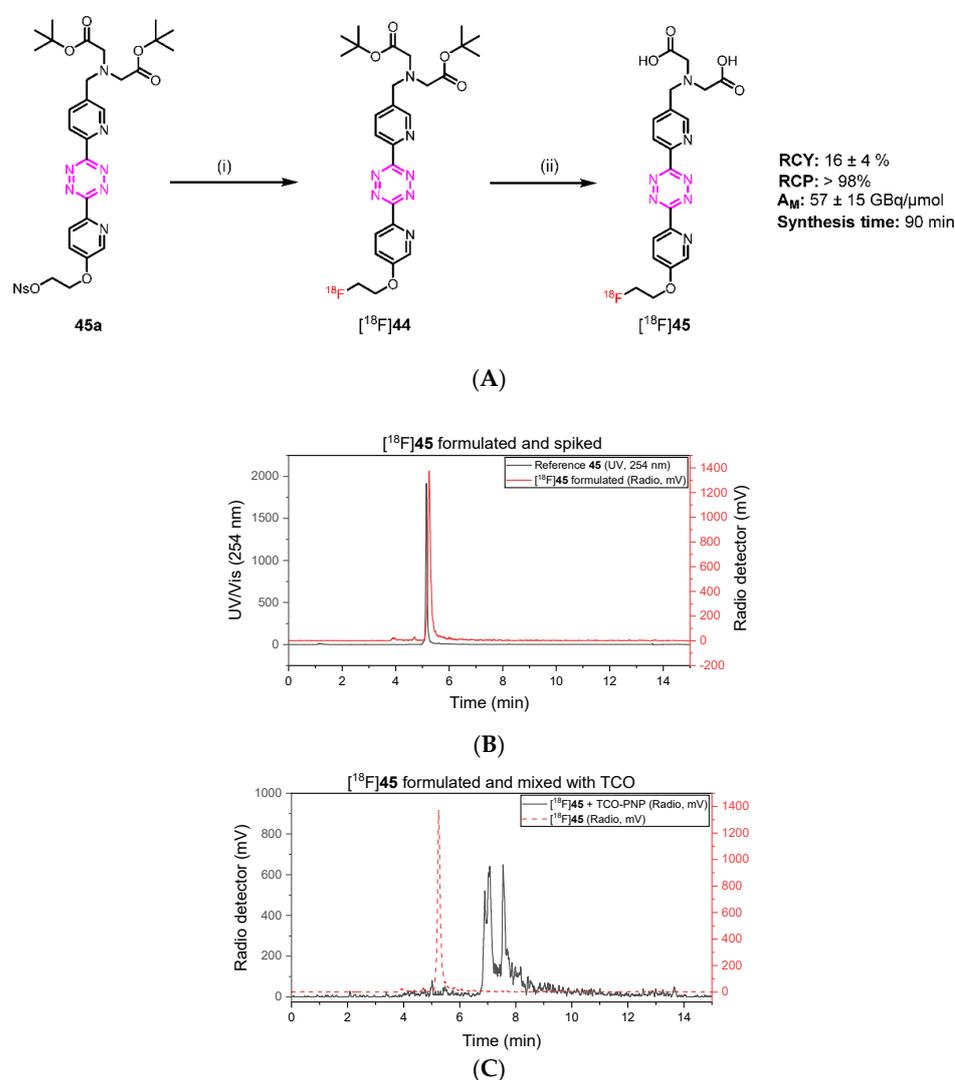


Figure 4. Radiolabeling of [^{18}F]45. **(A)** Reaction sequence. Reagents and conditions: (i) [^{18}F] Bu $_4$ NF/Bu $_4$ NOMs PO $_4^{3-}$, *t*-BuOH/DMSO, 100 °C, 5 min; (ii) TFA, CH $_3$ CN, 80 °C, 10 min. **(B)** Analytical chromatograms of formulated [^{18}F]45 in PBS (pH = 7.4) spiked with nonradioactive **45** for identification (rt: 5.20 min). **(C)** Analytical chromatogram of the reaction between [^{18}F]45 showing that the tetrazine core was intact. Analytical chromatogram of [^{18}F]15 formulated before TCO-PNP addition (red) and after TCO-PNP addition (black).

2.8. In Vivo Pretargeting PET Imaging with [¹⁸F]45

The ability of [¹⁸F]45 to act as a pretargeting imaging agent was evaluated in LS174T tumor-bearing mice. Mice were grouped and administered with either monoclonal anti-TAG72 antibody, CC49, or TCO-conjugated CC49 (CC49-TCO) 72 h prior to injection of [¹⁸F]45. PET/CT scans were acquired 1 h later, as well as image-derived uptake values from tumor, heart, and muscle tissue extracted by manually created regions of interests (Figure 5).

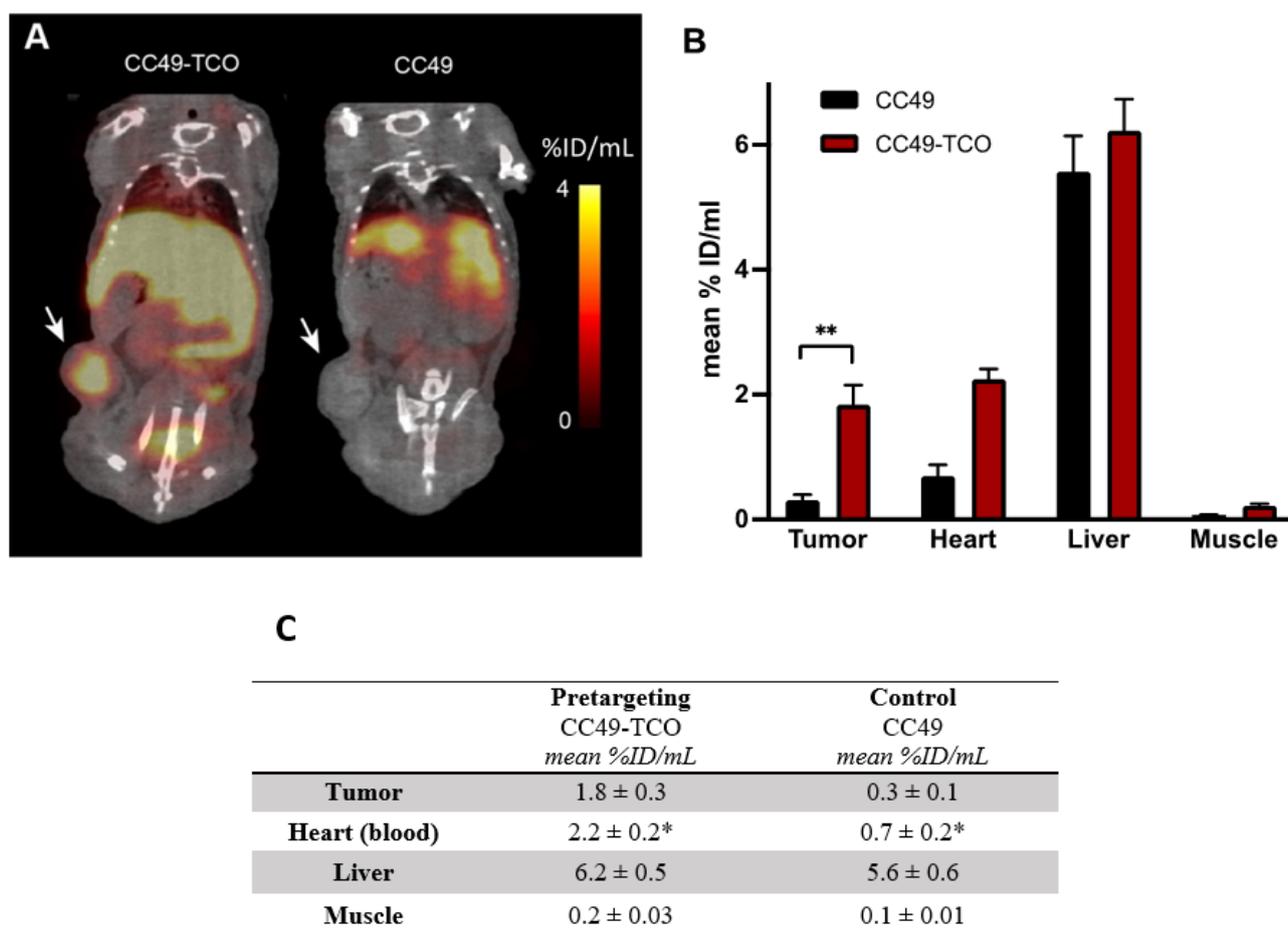


Figure 5. PET/CT imaging of [¹⁸F]45. (A) Images from PET/CT scans of [¹⁸F]45 1 h p.i. from mice pretreated with either CC49-TCO (left) or CC49 (right) 72 h earlier. Scale bar indicates mean percent of injected dose per gram (%ID/mL). White arrows indicate LS174T tumor xenografts. (B) Image-derived tissue uptake (mean %ID/mL) of [¹⁸F]45 1 h p.i. in mice administered with either CC49-TCO or CC49 72 h prior to [¹⁸F]45 injection. (C) Image-derived tissue uptake of [¹⁸F]45 in mean %ID/mL. All values are reported as mean ± S.E.M, *n* = 5/group. * Uptake in heart was considered as a surrogate for blood. ** *p* < 0.01 (Welch's *t*-test).

The tumors of the mice in the group receiving CC49-TCO had a significant higher uptake of [¹⁸F]45 (1.8 ± 0.3%ID/mL, mean ± SEM, *n* = 5) compared to the animals in the control group (0.3 ± 0.1%ID/mL, *n* = 5). Uptake in muscle tissue was generally low, resulting in a tumor-to-muscle ratio of 8.3 in animals pretargeted with CC49-TCO. In contrast, the uptake in blood (heart used as a surrogate) in this group of animals was high, resulting in a tumor-to-blood (T/B) ratio of 0.8. The tumor-to-liver (T/L) ratio was 0.3. Therefore, the uptake and target-to-background contrast found for [¹⁸F]45 were at comparable levels to previous findings [17,32,34]. However, there is a general need to improve the target-to-background contrast before clinical translation. This could either be achieved by developing primary and secondary vectors with improved performance,

thereby increasing the tumor uptake. Further, the use of a clearing agent could also potentially accelerate clearance of the primary vector and thus improve the T/B ratio in the future [14].

3. Materials and Methods

3.1. Chemistry

3.1.1. General

All reagents and solvents were purchased from commercial suppliers and used without further purification. Anhydrous tetrahydrofuran (THF) was obtained from an SG water solvent purification system (Pure Process Technology). Anhydrous dimethyl sulfoxide (DMSO), *N,N*-dimethylacetamide (DMA), MeCN, and pyridine were purchased from commercial suppliers and stored under argon. Reactions requiring anhydrous conditions were carried out under an inert atmosphere (nitrogen or argon) and using oven-dried glassware (152 °C). Syringes used to transfer anhydrous solvents or reagents were purged with argon prior to use. Other solvents were analytical or HPLC grade and were used as received. NMR spectra were acquired on a 600 MHz Bruker Avance III HD (600 MHz for ¹H and 151 MHz for ¹³C), a 400 MHz Bruker Avance II (400 MHz for ¹H, 376 MHz for ¹⁹F, and 101 MHz for ¹³C), and a 400 MHz Bruker Avance UltraShield (400 MHz for ¹H, 376 MHz for ¹⁹F, and 101 MHz for ¹³C) using chloroform-*d*, MeOD, or DMSO-*d*₆ as a deuterated solvent, and with the residual solvent as the internal reference. For all NMR experiments, the deuterated solvent signal was used as the internal lock. 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 were reprocessed in MestReNova software (version 12.0.22023) from the original FID files. Yields refer to isolated compounds estimated to be >90% pure as determined by ¹H NMR (25 °C) and analytical HPLC (Please refer to the Supplementary Materials). Analytical HPLC method: Thermo Fisher UltiMate 3000 with a C-18 column (Luna 5 μm C18(2) 100 Å, 150 mm × 4.6 mm). Eluents: A, H₂O with 0.1% TFA; B, MeCN with 0.1% TFA. Gradient from 100% A to 100% B for 12 min, back to 100% A for 3 min, flow rate 2 mL/min. Detection by UV absorption at λ = 254 nm on a UVD 170U detector. Thin-layer chromatography (TLC) was carried out on silica gel 60 F₂₅₄ plates from Merck (Germany). Visualization was accomplished by UV lamp (254 nm). Preparative HPLC was carried out on an UltiMate HPLC system (Thermo Scientific) consisting of an LPG-3200BX pump (10 mL/min), a Rheodyne 9725i injector, a 10 mL loop, a MWD-3000SD detector (254 nm), and an AFC-3000SD automated fraction collector, using a Gemini-NX C18 column (21.2 × 250 mm, 5 μm, 110 Å) (Phenomenex) equipped with a guard. Purifications were performed using linear gradients of 0.1% TFA in MilliQ-H₂O (A) and 0.1% TFA, 10% MilliQ-H₂O in MeCN (B). Data were acquired and processed using Chromeleon software v. 6.80. Semipreparative HPLC was performed on the same system using a Luna 5 μm C18 column (250 × 10 mm) with a flow rate of 3 mL/min. Automated flash column chromatography was performed on a CombiFlash NextGen 300+ system supplied by TeleDyne ISCO, equipped with RediSep silica-packed columns. Detection of the compounds was carried out by means of a UV-vis variable wavelength detector operating at 200 to 800 nm and by an evaporative light-scattering detector (ELSD). Solvent systems for separation were particular for each compound, but consisted of various mixtures of heptane, EtOAc, CH₂Cl₂, and MeOH. Microwave-assisted synthesis was carried out in a Biotage Initiator apparatus operating in single mode; the microwave cavity produced controlled irradiation at 2.45 GHz (Biotage AB, Uppsala, Sweden). The reactions were run in sealed vessels. These experiments were performed by employing magnetic stirring and a fixed hold time using variable power to reach (during 1–2 min) and then maintain the desired temperature in the vessel for the programmed time period. The temperature was monitored by an IR sensor focused on a point on the reactor vial glass. The IR sensor was calibrated to the internal solution's reaction temperature by the manufacturer. Mass spectra analysis was completed using

MS-Acquity-A: Waters Acquity UPLC with QDa-detector. CC49-TCO was kindly provided by Tagworks Pharmaceuticals, and it was obtained as previously described [14].

3.1.2. 2-Fluoroethyl 4-(1,2,4,5-tetrazin-3-yl)benzoate (**3**)

4-(1,2,4,5-Tetrazin-3-yl)benzoic acid (**10**)

The compound was synthesized as previously reported [42]. 4-Cyanobenzoic acid (0.3 g, 2.00 mmol), CH₂Cl₂ (0.12 mL, 2.00 mmol), sulfur (0.12 g, 0.5 mmol), and ethanol (4.0 mL) were mixed together in a microwave reaction vial. Hydrazine monohydrate (0.82 mL, 16.00 mmol) was added dropwise with stirring. The vessel was sealed, and the reaction mixture was heated to 50 °C for 24 h. The reaction was diluted with 3 mL of CH₂Cl₂, and sodium nitrite (1.44 g, 20.00 mmol) in 20 mL of H₂O was added dropwise to the mixture under cooling. Excess acetic acid (7 mL) was then added slowly, during which the solution turned bright red in color. The reaction mixture was extracted with CH₂Cl₂ (3 × 20 mL). The organic phase was dried over MgSO₄ and concentrated under reduced pressure. The resulting residue was purified using flash chromatography (CH₂Cl₂/MeOH 98/2) to give 0.08 g (20%) of the desired product as a pink solid. R_f = 0.31 (CH₂Cl₂/MeOH 95/5); ¹H NMR (400 MHz, DMSO) δ 13.32 (s, 1H), 10.66 (s, 1H), 8.62 (d, *J* = 8.5 Hz, 2H), 8.22 (d, *J* = 8.5 Hz, 2H); ¹³C NMR (101 MHz, DMSO) δ 166.67, 165.08, 158.24, 135.70, 134.32, 130.20, 127.97.

2-Fluoroethyl 4-(1,2,4,5-tetrazin-3-yl)benzoate (**3**)

Compound **10** (0.04 g, 0.20 mmol) and 1-fluoro-2-iodoethane (0.05 g, 0.30 mmol) were dissolved in 2 mL dry DMF, and DIPEA (0.10 mL, 0.60 mmol) was added. The reaction was left at 70 °C overnight. The reaction was diluted with CH₂Cl₂ (15 mL) and washed with a saturated aqueous solution of NH₄Cl (10 mL) and H₂O (2 × 10 mL). The organic phase was dried over MgSO₄ and concentrated under reduced pressure. Purification by flash chromatography (85/15 heptane/EtOAc) afforded 0.04 g (81%) of **3** as a red solid. R_f = 0.23 (heptane/EtOAc 80/20); ¹H NMR (600 MHz, CDCl₃) δ 10.21 (s, 1H), 8.65 (d, *J* = 8.6 Hz, 2H), 8.23 (d, *J* = 8.5 Hz, 2H), 4.89–4.72 (m, 1H), 4.69–4.64 (m, 1H), 4.63–4.57 (m, 1H), 4.57–4.50 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 165.92, 165.50, 157.99, 135.75, 133.63, 130.58, 128.27, 81.26 (d, *J* = 171.1 Hz), 64.30 (d, *J* = 20.1 Hz).

3.1.3. 2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl 4-(1,2,4,5-tetrazin-3-yl)benzoate (**3a**)

2-Hydroxyethyl 4-(1,2,4,5-tetrazin-3-yl)benzoate (**11**)

Compound **10** (0.03 g, 0.15 mmol) and 2-bromoethanol (0.02 mL, 0.22 mmol) were dissolved in 3 mL dry DMF, DIPEA (0.08 mL, 0.44 mmol) was dissolved in 1 mL DMF and added dropwise, and the reaction was left at 70 °C. After the reaction was completed, it was cooled down, diluted with water (30 mL) and extracted with CH₂Cl₂ (3 × 20 mL). The crude was purified by flash chromatography (60/40 heptane/EtOAc) to give 0.03 g (82%) of the desired product as a red solid. R_f = 0.24 (heptane/EtOAc 70/30); ¹H NMR (600 MHz, CDCl₃) δ 10.28 (s, 1H), 8.72 (d, *J* = 8.1 Hz, 2H), 8.29 (d, *J* = 8.1 Hz, 2H), 4.54 (t, *J* = 4.6 Hz, 2H), 4.02 (t, *J* = 4.7 Hz, 2H), 2.56–1.97 (m, 1H), ¹³C NMR (151 MHz, CDCl₃) δ 166.04, 165.93, 158.00, 135.68, 133.87, 130.55, 128.28, 67.10, 61.31.

2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl 4-(1,2,4,5-tetrazin-3-yl)benzoate (**3a**)

To a solution of compound **11** (0.03 g, 0.12 mmol) and DIPEA (0.04 mL, 0.24 mmol) in CH₂Cl₂ (10 mL) were added nosyl chloride (0.041 g, 0.18 mmol) and DMAP (0.001 g, 0.01 mmol). The reaction was stirred at room temperature for 1 h. The solvent was removed under reduced pressure. Purification by flash chromatography (80/20 heptane/EtOAc) afforded 0.035 (67%) of **3a** as a red solid. R_f = 0.38 (heptane/EtOAc 70/30); ¹H NMR (600 MHz, CDCl₃) δ 10.31 (s, 1H), 8.71 (d, *J* = 8.5 Hz, 2H), 8.31 (d, *J* = 8.8 Hz, 2H), 8.16 (d, *J* = 8.5 Hz, 2H), 8.14–8.09 (m, 2H), 4.64–4.58 (m, 2H), 4.58–4.53 (m, 2H); ¹³C NMR (151 MHz, CDCl₃) δ 165.77, 165.07, 158.06, 150.73, 141.65, 136.14, 132.92, 130.48, 129.17, 128.27, 124.50, 68.72, 62.28.

3.1.4. N-(2-Fluoroethyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (**4**)

4-Cyano-N-(2-fluoroethyl)benzamide (**12**)

To a solution of 4-cyanobenzoic acid (0.73 g, 5.00 mmol) in CH₃CN (20 mL) was added CDI (1.21 g, 7.50 mmol). The mixture was stirred at room temperature for 45 min, before addition of 2-fluoroethylamine hydrochloride (0.55 g, 5.50 mmol) and Et₃N (2.09 mL, 15.00 mmol). The reaction mixture was stirred for 2 h. Water (30 mL) was added, and the mixture was extracted with EtOAc (3 × 20 mL). The organic phase was dried over MgSO₄ and concentrated under reduced pressure to give 0.65 g (68%) of the desired compound as a white solid. R_f = 0.26 (heptane/EtOAc 60/40); ¹H NMR (400 MHz, DMSO) δ 8.96 (s, 1H), 8.02 (d, *J* = 8.7 Hz, 2H), 7.97 (d, *J* = 8.6 Hz, 2H), 4.61 (t, *J* = 5.1 Hz, 1H), 4.50 (t, *J* = 5.1 Hz, 1H), 3.62 (q, *J* = 5.2 Hz, 1H), 3.56 (q, *J* = 5.2 Hz, 1H); ¹³C NMR (101 MHz, DMSO) δ 165.61, 138.57, 132.92, 128.55, 114.17, 82.49 (d, *J* = 165.8 Hz), 40.57 (d, *J* = 20.9 Hz).

N-(2-Fluoroethyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (**4**)

The compound was obtained from **12** (0.60 g, 3.12 mmol) as described for compound **10**. Purification by flash chromatography (60/40 heptane/EtOAc) afforded 0.36 g (47%) of **4** as a red solid. R_f = 0.38 (heptane/EtOAc 50/50); ¹H NMR (400 MHz, DMSO) δ 10.65 (s, 1H), 8.95 (t, *J* = 5.5 Hz, 1H), 8.60 (d, *J* = 8.6 Hz, 2H), 8.15 (d, *J* = 8.6 Hz, 2H), 4.65 (t, *J* = 5.1 Hz, 1H), 4.53 (t, *J* = 5.1 Hz, 1H), 3.66 (q, *J* = 5.3 Hz, 1H), 3.59 (q, *J* = 5.2 Hz, 1H); ¹³C NMR (101 MHz, DMSO) δ 166.24, 165.59, 158.70, 138.21, 134.81, 128.75, 128.23, 82.57 (d, *J* = 165.8 Hz), 40.56 (d, *J* = 16.2 Hz).

3.1.5. 2-(4-(1,2,4,5-Tetrazin-3-yl)benzamido)ethyl 4-nitrobenzenesulfonate (**4a**)

4-Cyano-N-(2-hydroxyethyl)benzamide (**13**)

The compound was obtained from 4-cyanobenzoic acid (1.17 g, 8.00 mmol) and ethanolamine (4.82 mL, 80.00 mmol) as described for compound **12**. Purification by flash chromatography (CH₂Cl₂/MeOH 95/5) afforded 1.37 g (90%) of the desired compound as a colorless oil. R_f = 0.22 (CH₂Cl₂/MeOH 95/5); ¹H NMR (400 MHz, DMSO) δ 8.70 (t, *J* = 5.6 Hz, 1H), 8.01 (d, *J* = 8.6 Hz, 2H), 7.96 (d, *J* = 8.6 Hz, 2H), 4.75 (t, *J* = 5.6 Hz, 1H), 3.53 (q, *J* = 5.9 Hz, 2H), 3.39–3.32 (m, 2H); ¹³C NMR (101 MHz, DMSO) δ 165.38, 139.03, 132.83, 128.54, 118.83, 113.94, 60.01, 42.82.

N-(2-Hydroxyethyl)-4-(1,2,4,5-tetrazin-3-yl)benzamide (**14**)

The compound was obtained from **13** (1.00 g, 5.25 mmol) as described for compound **10**. Purification by flash chromatography (CH₂Cl₂/MeOH 97/3) afforded 0.39 g (30%) of the desired compound as a colorless oil. R_f = 0.31 (CH₂Cl₂/MeOH 95/5); ¹H NMR (600 MHz, DMSO) δ 10.64 (s, 1H), 8.68 (t, *J* = 5.6 Hz, 1H), 8.61–8.56 (m, 2H), 8.16–8.10 (m, 2H), 4.75 (t, *J* = 5.6 Hz, 1H), 3.56 (q, *J* = 6.1 Hz, 2H), 3.38 (q, *J* = 6.0 Hz, 2H); ¹³C NMR (151 MHz, DMSO) δ 166.00, 165.61, 158.71, 158.68, 138.66, 128.72, 128.16, 60.13, 42.80.

2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-4,5-dihydrooxazole

The compound was obtained from **14** (0.05 g, 0.21 mmol) as described for compound **3a**. An intramolecular substitution occurred during the reaction, giving this side product. Purification by flash chromatography (60/40 heptane/EtOAc) afforded 0.35 g (75%) of this side product as a red solid. R_f = 0.41 (heptane/EtOAc 50/50); ¹H NMR (600 MHz, CDCl₃) δ 10.18 (s, 1H), 9.09–8.44 (m, 2H), 8.11 (d, *J* = 8.1 Hz, 2H), 4.43 (t, *J* = 9.5 Hz, 2H), 4.06 (t, *J* = 9.5 Hz, 2H); ¹³C NMR (151 MHz, CDCl₃) δ 166.07, 163.83, 157.88, 133.94, 132.05, 129.06, 128.22, 67.91, 55.17.

3.1.6. 2-Fluoroethyl 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetate (**5**)

2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)acetic acid (**15**)

The compound was obtained from 2-(4-cyanophenyl)acetic acid (0.97 g, 6.00 mmol) as described for compound **10**. Purification by flash chromatography (98/2 CH₂Cl₂/MeOH) to afford 0.36 g (28%) of the desired compound as a pink solid. R_f = 0.19 (95/5 CH₂Cl₂/MeOH); ¹H NMR (400 MHz, MeOD) δ 10.33 (s, 1H), 8.56 (d, *J* = 8.4 Hz, 2H), 7.59 (d, *J* = 8.3 Hz, 2H), 3.78 (s, 2H); ¹³C NMR (101 MHz, MeOD) δ 173.27, 166.24, 157.83, 140.15, 130.69, 130.13, 127.78, 40.39.

2-Fluoroethyl 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetate (5)

The compound was obtained from **15** (0.07 g, 0.32 mmol) as described for compound **3**. Purification by flash chromatography (80/20 heptane/EtOAc) afforded 0.042 g (49%) of **5** as a red solid. R_f = 0.20 (80/20 heptane/EtOAc); ¹H NMR (400 MHz, CDCl₃) δ 10.14 (s, 1H), 8.53 (d, *J* = 8.4 Hz, 2H), 7.47 (d, *J* = 8.1 Hz, 2H), 4.63–4.57 (m, 1H), 4.51–4.46 (m, 1H), 4.37–4.32 (m, 1H), 4.30–4.25 (m, 1H), 3.74 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 170.58, 166.25, 157.79, 139.06, 130.62, 130.38, 128.56, 81.14 (d, *J* = 170.9 Hz), 63.96 (d, *J* = 20.2 Hz), 41.01.

3.1.7. 2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetate (5a)**2-Hydroxyethyl 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetate (16)**

The compound was obtained from **15** (0.07 g, 0.32 mmol) as described for compound **11**. Purification by flash chromatography (80/20 heptane/EtOAc) afforded 0.05 g (59%) of the desired product as a red solid. R_f = 0.17 (60/40 heptane/EtOAc); ¹H NMR (400 MHz, CDCl₃) δ 10.14 (s, 1H), 8.51 (d, *J* = 8.4 Hz, 2H), 7.46 (d, *J* = 8.3 Hz, 2H), 4.24–4.17 (m, 2H), 3.81–3.74 (m, 2H), 3.73 (s, 2H), 1.91 (s, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 171.09, 166.22, 157.78, 139.23, 130.58, 130.36, 128.55, 66.68, 61.06, 41.13.

2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl 2-(4-(1,2,4,5-tetrazin-3-yl)phenyl)acetate (5a)

The compound was obtained from **16** (0.50 g, 0.19 mmol) as described for compound **3a**. Purification by flash chromatography (75/25 heptane/EtOAc) afforded 0.05 g (58%) of **5a** as a red solid. R_f = 0.27 (heptane/EtOAc 60/40); ¹H NMR (400 MHz, CDCl₃) δ 10.16 (s, 1H), 8.51 (d, *J* = 8.2 Hz, 2H), 8.30 (d, *J* = 8.8 Hz, 2H), 8.02 (d, *J* = 8.8 Hz, 2H), 7.43 (d, *J* = 8.2 Hz, 2H), 4.30 (s, 4H), 3.69 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 170.30, 157.84, 150.86, 141.58, 138.66, 130.76, 130.35, 129.20, 128.56, 124.52, 68.35, 62.00, 40.86.

3.1.8. 2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-N-(2-fluoroethyl)acetamide (6)**2-(4-Cyanophenyl)-N-(2-fluoroethyl)acetamide (17)**

The compound was obtained from 2-(4-cyanophenyl)acetic acid (0.5 g, 3.10 mmol) as described for compound **12** [17]. Concentration under reduced pressure afforded 0.46 g (72%) of the desired product as a white solid. R_f: 0.35 (heptane/EtOAc 30/70); ¹H NMR (400 MHz, CDCl₃) δ 7.57 (d, *J* = 8.3 Hz, 2H), 7.34 (d, *J* = 8.2 Hz, 2H), 5.88 (s, 1H), 4.47 (t, *J* = 4.8 Hz, 1H), 4.35 (t, *J* = 4.8 Hz, 1H), 3.59–3.49 (m, 3H), 3.46 (dt, *J* = 5.8, 4.7 Hz, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 169.58, 140.02, 132.56, 130.10, 118.58, 111.32, 82.48 (d, *J* = 166.7 Hz), 43.36, 40.23 (d, *J* = 19.5 Hz).

2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-N-(2-fluoroethyl)acetamide (6)

The compound was obtained from **17** (0.40 g, 1.94 mmol) as described for compound **3** [17]. Purification by flash chromatography (30/70 heptane/EtOAc) afforded 0.21 g (41%) of **6** as a red solid. R_f = 0.22 (heptane/EtOAc 40/60); ¹H NMR (400 MHz, DMSO) δ 10.58 (s, 1H), 8.48–8.40 (m, 3H), 7.57 (d, *J* = 8.2 Hz, 2H), 4.51 (t, *J* = 5.0 Hz, 1H), 4.39 (t, *J* = 5.0 Hz, 1H), 3.62 (s, 2H), 3.43 (q, *J* = 5.3 Hz, 1H), 3.36 (q, *J* = 5.2 Hz, 1H); ¹³C NMR (101 MHz, DMSO) δ 170.27, 165.92, 158.54, 142.02, 130.61, 130.53, 128.15, 82.90 (d, *J* = 165.0 Hz), 42.56, 40.56 (d, *J* = 15.8 Hz).

3.1.9. 2-(2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)acetamido)ethyl 4-nitrobenzenesulfonate (6a)**2-(4-Cyanophenyl)-N-(2-hydroxyethyl)acetamide (18)**

The compound was obtained from 2-(4-cyanophenyl)acetic acid (2.1 g, 13.03 mmol) as described for compound **13**. Recrystallization from EtOAc afforded 1.60 g (60%) of the desired product as a white solid. R_f = 0.15 (heptane/EtOAc 20/80); ¹H NMR (400 MHz, DMSO) δ 8.15 (s, 1H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.46 (d, *J* = 8.0 Hz, 2H), 4.68 (t, *J* = 5.4 Hz, 1H), 3.54 (s, 2H), 3.40 (q, *J* = 5.8 Hz, 2H), 3.12 (q, *J* = 5.9 Hz, 2H); ¹H NMR (400 MHz, DMSO) δ 8.15 (s, 1H), 7.76 (d, *J* = 8.2 Hz, 2H), 7.46 (d, *J* = 8.0 Hz, 2H), 4.68 (t, *J* = 5.4 Hz, 1H), 3.54 (s, 2H), 3.40 (q, *J* = 5.8 Hz, 2H), 3.12 (q, *J* = 5.9 Hz, 2H).

2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)-N-(2-hydroxyethyl)acetamide (19)

The compound was obtained from **18** (0.82 g, 4.00 mmol) as described for compound **3**. Purification by flash chromatography (98/2 CH₂Cl₂/MeOH) and recrystal-

lization from EtOAc afforded 0.33 g (32%) of the desired product as a pink solid. $R_f = 0.31$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5); $^1\text{H NMR}$ (400 MHz, DMSO) δ 10.31 (s, 1H), 8.54 (d, $J = 8.2$ Hz, 2H), 7.58 (d, $J = 8.1$ Hz, 2H), 3.67 (s, 2H), 3.62 (t, $J = 5.8$ Hz, 2H), 3.36–3.32 (m, 2H); $^{13}\text{C NMR}$ (101 MHz, DMSO) δ 171.23, 165.43, 157.02, 140.25, 129.84, 129.01, 127.04, 59.30, 41.46, 40.99.

2-(2-(4-(1,2,4,5-Tetrazin-3-yl)phenyl)acetamido)ethyl 4-nitrobenzenesulfonate (6a)

The compound was obtained from **19** (0.06 g, 0.23 mmol) as described for compound **3a**. Purification by flash chromatography (99/1 $\text{CH}_2\text{Cl}_2/\text{MeOH}$) afforded 0.08 g (83%) of **3a** as a red solid. $R_f = 0.31$ (heptane/EtOAc 60/40); $^1\text{H NMR}$ (400 MHz, DMSO) δ 10.51 (s, 1H), 8.46 (d, $J = 8.9$ Hz, 2H), 8.39 (d, $J = 8.6$ Hz, 2H), 8.29 (d, $J = 8.9$ Hz, 2H), 7.69 (d, $J = 8.6$ Hz, 2H), 5.83 (s, 1H), 4.29 (t, $J = 7.0$ Hz, 2H), 4.10 (t, $J = 7.0$ Hz, 2H), 3.31 (s, 2H); $^{13}\text{C NMR}$ (101 MHz, DMSO) δ 165.77, 158.22, 151.32, 149.36, 141.57, 140.68, 129.72, 128.22, 128.11, 128.07, 125.43, 86.21, 67.35, 47.47.

3.1.10. 3-(4-(2-Fluoroethoxy)phenyl)-1,2,4,5-tetrazine (7)

4-(2-Fluoroethoxy)benzotrile (20)

To a solution of 4-hydroxybenzotrile (0.6 g, 5.00 mmol) and K_2CO_3 (1.38 g, 10.00 mmol) in CH_3CN (20 mL) was added 1-fluoro-2-iodoethane (1.04 g, 6.00 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 (3×50 mL), washed with brine (50 mL), dried over MgSO_4 , filtered, and concentrated under reduced pressure. Purification by flash chromatography (heptane/EtOAc 80/20) afforded 0.8 g (97%) of the desired compound as a yellow oil. $R_f = 0.37$ (heptane/EtOAc 70/30); $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 7.62 (d, $J = 8.9$ Hz, 1H), 7.01 (d, $J = 8.9$ Hz, 1H), 5.05–4.79 (m, 1H), 4.77–4.71 (m, 1H), 4.36–4.29 (m, 1H), 4.29–4.22 (m, 1H); $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 161.69, 134.06, 119.03, 115.33, 104.59, 81.48 (d, $J = 171.7$ Hz), 67.37 (d, $J = 20.5$ Hz).

3-(4-(2-Fluoroethoxy)phenyl)-1,2,4,5-tetrazine (7)

The compound was obtained from **20** (0.73 g, 4.42 mmol) as described for compound **3**. Purification by flash chromatography (heptane/EtOAc 90/10) afforded 0.36 g (37%) of the desired compound as a red oil. $R_f = 0.33$ (heptane/EtOAc 80/20); $^1\text{H NMR}$ (400 MHz, CDCl_3) δ 10.07 (s, 1H), 8.53 (d, $J = 8.9$ Hz, 2H), 7.06 (d, $J = 8.9$ Hz, 2H), 4.89–4.78 (m, 1H), 4.74–4.62 (m, 1H), 4.43–4.29 (m, 1H), 4.27–4.16 (m, 1H); $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 166.06, 162.56, 157.40, 130.26, 124.56, 115.38, 81.63 (d, $J = 171.5$ Hz), 67.29 (d, $J = 20.6$ Hz).

3.1.11. 2-(4-(1,2,4,5-Tetrazin-3-yl)phenoxy)ethyl 4-nitrobenzenesulfonate (7a)

4-(2-Hydroxyethoxy)benzotrile (21)

To a solution of 4-hydroxybenzotrile (1.42 g, 12.00 mmol) and K_2CO_3 (8.29 g, 60.00 mmol) in CH_3CN (20 mL) was added 2-bromoethanol (2.55 mL, 36.00 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 (3×50 mL), washed with brine (50 mL), dried over MgSO_4 , filtered, and concentrated under reduced pressure. Purification by flash chromatography (heptane/EtOAc 40/60) afforded 1.41 g (72%) of the desired compound as a yellow oil. $R_f = 0.18$ (heptane/EtOAc 50/50); $^1\text{H NMR}$ (400 MHz, DMSO) δ 7.76 (d, $J = 8.8$ Hz, 2H), 7.11 (d, $J = 8.8$ Hz, 2H), 4.92 (t, $J = 5.5$ Hz, 1H), 4.09 (t, $J = 5.0$ Hz, 2H), 3.73 (q, $J = 5.0$ Hz, 2H); $^{13}\text{C NMR}$ (101 MHz, DMSO) δ 162.70, 134.63, 119.64, 116.05, 103.15, 70.56, 59.79.

2-(4-(1,2,4,5-Tetrazin-3-yl)phenoxy)ethan-1-ol (22)

The compound was obtained from **21** (0.98 g, 6.00 mmol) as described for compound **3**. Purification by flash chromatography (heptane/EtOAc 50/50) afforded 0.71 g (54%) of the desired product as a red solid. $R_f = 0.27$ (heptane/EtOAc 50/50); $^1\text{H NMR}$ (400 MHz, DMSO) δ 10.50 (s, 1H), 8.47 (d, $J = 9.0$ Hz, 2H), 7.23 (d, $J = 9.0$ Hz, 2H), 4.94 (t, $J = 5.5$ Hz, 1H), 4.15 (t, $J = 4.9$ Hz, 2H), 3.78 (q, $J = 5.2$ Hz, 2H); $^{13}\text{C NMR}$ (101 MHz, DMSO) δ 165.65, 163.10, 158.16, 130.10, 124.35, 115.95, 70.44, 59.92.

2-(4-(1,2,4,5-Tetrazin-3-yl)phenoxy)ethyl 4-nitrobenzenesulfonate (7a)

Compound **21** (0.10 g, 0.46 mmol) was mixed with nitrobenzenesulfonyl chloride (0.15 g, 0.69 mmol) and dissolved in 4 mL dry CH₂Cl₂ under argon. A mixture of DIPEA (0.33 mL, 1.83 mmol) and DMAP (5 mg, 0.04 mmol) in 1 mL dry CH₂Cl₂ was added at 0 °C under argon. The reaction was slowly heated to room temperature and left for 1 h. The reaction was diluted with 10 mL CH₂Cl₂ and washed with 20 mL NH₄Cl (sat.) and H₂O (2 × 20 mL). The organic phase was dried over N₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography (heptane/EtOAc 60/40) afforded 0.12 g (65%) of the desired product as a red solid. R_f = 0.41 (heptane/EtOAc 50/50); ¹H NMR (400 MHz, DMSO) δ 10.52 (s, 1H), 8.97–8.31 (m, 5H), 8.33–7.87 (m, 2H), 7.11 (d, *J* = 8.9 Hz, 2H), 4.97–4.50 (m, 2H), 4.48–4.06 (m, 2H); ¹³C NMR (101 MHz, DMSO) δ 165.57, 161.88, 158.28, 154.85, 147.71, 130.25, 127.38, 125.26, 123.79, 116.05, 75.01, 67.33.

3.1.12. N-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-2-fluoroethan-1-amine (**9**)

4-((2-Fluoroethyl)amino)methylbenzonitrile (**23**)

To a solution of 4-(bromomethyl)benzonitrile (0.78 g, 4.00 mmol) in CH₃CN (40 mL) was added K₂CO₃ (0.33 g, 24.0 mmol) and 2-fluoroethylamine hydrochloride (0.16 g, 16.0 mmol). The mixture was stirred at room temperature overnight. The solvent was removed under reduced pressure, and the residue was diluted with water (20 mL) and extracted with EtOAc. The combined organic layer was washed with brine, dried over MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography using EtOAc (heptane/EtOAc 50/50) in heptane to afford 0.54 g (76%) of **23** as a colorless oil. R_f = 0.24 (heptane/EtOAc 40/60). ¹H NMR (400 MHz, CDCl₃) δ 7.55 (d, *J* = 8.2 Hz, 2H), 7.40 (d, *J* = 8.0 Hz, 2H), 4.63–4.48 (m, 1H), 4.47–4.37 (m, 1H), 3.84 (s, 2H), 2.93–2.84 (m, 1H), 2.84–2.72 (m, 1H), 1.65 (s, 1H). ¹³C NMR (101 MHz, CDCl₃) δ 145.6, 132.3, 128.6, 118.9, 110.9, 83.5 (d, *J* = 165.5 Hz), 53.1, 49.1 (d, *J* = 19.7 Hz)

Tert-butyl 4-cyanobenzyl(2-fluoroethyl)carbamate (**25**)

To a solution of **23** (540 mg, 3.03 mmol) and Et₃N (1.27 mL, 9.09 mmol) in CH₂Cl₂ (40 mL) was added Boc₂O (790 mg, 3.63 mmol), and the mixture was stirred at room temperature for 12 h. The solution was washed with water and saturated K₂CO₃ solution, dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash column chromatography using (heptane/EtOAc 70/30) to afford 0.710 g (84%) of the desired product as a colorless oil (mixture of rotamers). R_f = 0.42 (heptane/EtOAc 80/20). ¹H NMR (400 MHz, CDCl₃) δ 7.55 (d, *J* = 7.8 Hz, 2H), 7.27 (d, *J* = 7.8 Hz, 2H), 4.79–4.10 (m, 4H), 3.62–3.28 (m, 2H), 1.96–1.05 (m, 9H). ¹³C NMR (101 MHz, CDCl₃) δ 155.4, 144.2, 143.8, 132.4, 128.1, 127.5, 118.7, 111.1, 83.2 (d, *J* = 168.2 Hz), 82.7 (d, *J* = 170.5 Hz), 52.1, 51.2, 47.7, 28.3.

Tert-butyl 4-(1,2,4,5-tetrazin-3-yl)benzyl(2-fluoroethyl)carbamate (**27**)

The compound was obtained from **25** (0.68 g, 2.44 mmol) as described for compound **3**. Purification by flash chromatography (heptane/EtOAc 80/20) afforded 0.18 g (22%) of the desired product as a red solid (mixture of rotamers). R_f = 0.21 (heptane/EtOAc 80/20); ¹H NMR (400 MHz, CDCl₃) δ 10.23 (s, 1H), 8.62 (d, *J* = 7.8 Hz, 2H), 7.49 (d, *J* = 7.8 Hz, 2H), 4.76–4.42 (m, 4H), 3.83–3.38 (m, 2H), 1.64–1.38 (m, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 166.32, 157.77, 155.51, 144.25, 132.37, 130.58, 128.55, 127.86, 83.46, 83.17 (d, *J* = 165.4 Hz), 82.67 (d, *J* = 170.4 Hz), 80.63, 52.07, 51.07, 47.49, 28.36.

N-(4-(1,2,4,5-tetrazin-3-yl)benzyl)-2-fluoroethan-1-amine (**9**)

To a solution of **27** (0.10 mg, 0.30 mmol) in dioxane (10 mL) was added a solution of HCl in dioxane (4.0 M, 3.0 mL). The mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The obtained solid was washed with Et₂O to afford 0.07 g (86%) of **9** as hydrochloride salt. ¹H NMR (400 MHz, DMSO-*d*₆) δ 10.64 (s, 1H), 9.87 (s, 2H), 8.55 (d, *J* = 8.3 Hz, 2H), 7.89 (d, *J* = 8.3 Hz, 2H), 4.89 (t, *J* = 4.6 Hz, 1H), 4.77 (t, *J* = 4.6 Hz, 1H), 4.35 (s, 2H), 3.38 (t, *J* = 4.7 Hz, 1H), 3.31 (t, *J* = 4.7 Hz, 1H). ¹³C NMR (101 MHz, DMSO-*d*₆) δ 165.7, 158.7, 137.0, 132.8, 131.6, 128.4, 80.0 (d, *J* = 165.3 Hz), 50.1, 47.1 (d, *J* = 20.0 Hz).

3.1.13. 2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)(*tert*-butoxycarbonyl)amino)ethyl 4-nitro Benzenesulfonate (**9a**)

4-(((2-Hydroxyethyl)amino)methyl)benzonitrile (**24**)

The compound was obtained from 4-(bromomethyl)benzonitrile (2.00 g, 10.20 mmol) and ethanolamine (12.30 mL, 200.00 mmol) as described for compound **23**. Concentration under reduced pressure afforded 1.65 g (92%) of the desired compound as a white solid. R_f = 0.15 (90/10 CH₂Cl₂/MeOH); ¹H NMR (400 MHz, CDCl₃) δ 7.62–7.52 (m, 2H), 7.46–7.37 (m, 2H), 3.88–3.77 (m, 2H), 3.69–3.59 (m, 2H), 2.79–2.70 (m, 2H), 2.58–2.23 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 145.76, 132.22, 128.67, 118.88, 110.71, 60.91, 53.07, 50.78.

Tert-butyl (4-cyanobenzyl)(2-hydroxyethyl)carbamate (**26**)

The compound was obtained from **24** (1.62 g, 9.19 mmol) as described for compound **25**. Purification by flash chromatography (heptane/EtOAc 60/40) afforded 2.36 g (93%) of the desired product as a colorless oil (rotamers mixture). R_f = 0.4 (heptane/EtOAc 50/50); ¹H NMR (600 MHz, CDCl₃) δ 7.63 (d, *J* = 7.9 Hz, 2H), 4.55–4.52 (m, 2H), 3.74 (s, 2H), 3.51–2.94 (m, 2H), 2.69 (s, 1H), 1.76–0.53 (m, 9H); ¹³C NMR (151 MHz, CDCl₃) δ 156.91, 144.11, 132.43, 128.02, 127.50, 118.72, 111.19, 81.02, 62.17, 61.46, 52.10, 51.17, 50.37, 49.51, 28.32.

Tert-butyl (4-(1,2,4,5-tetrazin-3-yl)benzyl)(2-hydroxyethyl)carbamate (**28**)

The compound was obtained from **26** (2.32 g, 8.32 mmol) as described for compound **3**. Purification by flash chromatography (80/20 heptane/EtOAc) to give 1.07 g (39%) of the desired product as a red oil (rotamers mixture). R_f = 0.21 (heptane/EtOAc 70/30); ¹H NMR (400 MHz, CDCl₃) δ 10.17 (s, 1H), 8.53 (d, *J* = 8.0 Hz, 2H), 7.42 (d, *J* = 8.1 Hz, 2H), 4.57 (s, 2H), 3.71 (d, *J* = 5.8 Hz, 2H), 3.46–3.41 (m, 2H), 3.23 (q, *J* = 5.4 Hz, 1H), 1.39 (s, 9H); ¹³C NMR (101 MHz, CDCl₃) δ 166.23, 157.75, 156.89, 144.17, 132.36, 130.55, 128.52, 127.89, 80.75, 62.36, 61.76, 51.98, 51.12, 50.13, 49.44, 28.34.

3-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)oxazolidin-2-one

The compound was obtained from **28** (0.06 g, 0.18 mmol) as described for compound **3a**. An intramolecular substitution occurred during the reaction giving this side product. Purification by flash chromatography (50/50 heptane/EtOAc) afforded 0.035 (67%) of the side product as a red solid. R_f = 0.21 (heptane/EtOAc 50/50); ¹H NMR (400 MHz, CDCl₃) δ 10.16 (s, 1H), 8.55 (d, *J* = 8.3 Hz, 2H), 7.47 (d, *J* = 8.3 Hz, 2H), 4.46–3.83 (m, 2H), 3.45 (dd, *J* = 8.7, 7.3 Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 166.16, 158.59, 157.88, 141.30, 131.38, 128.96, 128.83, 61.87, 48.23, 44.21.

3.1.14. N-(4-(1,2,4,5-Tetrazin-3-yl)benzyl)-2-fluoro-N-tritylethan-1-amine (**30**)

Compound **9** (0.05 g, 0.18 mmol) was mixed with trityl chloride (0.05 g, 0.20 mmol) and dissolved in 4 mL dry CH₂Cl₂ under argon. A mixture of Et₃N (0.08 mL, 0.55 mmol) in 1 mL dry CH₂Cl₂ was added at 0 °C under argon. The reaction was slowly heated to room temperature and left for 1 h. The reaction was diluted with 10 mL CH₂Cl₂ and washed with NH₄Cl (sat.) 20 mL and H₂O (2 × 20 mL). The organic phase was dried over N₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography (heptane/EtOAc 80/20) afforded 0.08 g (78%) as a red solid. R_f = 0.41 (heptane/EtOAc 80/20); ¹H NMR (600 MHz, CDCl₃) δ 10.13 (s, 1H), 8.57 (d, *J* = 8.4 Hz, 2H), 7.77 (d, *J* = 8.4 Hz, 2H), 7.74–7.55 (m, 6H), 7.32–7.23 (m, 6H), 7.16–7.10 (m, 3H), 3.99 (t, *J* = 6.0 Hz, 1H), 3.91 (t, *J* = 6.1 Hz, 1H), 3.69 (s, 2H), 2.92–2.55 (m, 2H); ¹³C NMR (151 MHz, CDCl₃) δ 166.46, 157.73, 147.36, 143.10, 130.32, 129.32, 128.56, 128.43, 127.93, 127.87, 126.46, 81.94 (d, *J* = 168.6 Hz), 79.00, 57.49, 54.66 (d, *J* = 22.8 Hz).

3.1.15. 2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)(trityl)amino)ethyl 4-nitrobenzenesulfonate (**9b**)

2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)amino)ethan-1-ol (**29**)

To a solution of **28** (0.99 g, 2.98 mmol) in dioxane (10 mL) was added a solution of HCl in dioxane (4.0 M, 7.7 mL). The mixture was stirred at room temperature for 2 h and then concentrated under reduced pressure. The obtained solid was washed with Et₂O to afford 0.72 g (90%) of **29** as hydrochloride salt. ¹H NMR (400 MHz, DMSO) δ 10.64 (s, 1H), 9.50 (s,

3H), 8.96–8.31 (m, 2H), 8.09–7.60 (m, 2H), 4.32 (t, $J = 5.7$ Hz, 2H), 3.73 (t, $J = 5.4$ Hz, 2H), 3.01 (q, $J = 5.6$ Hz, 2H); ^{13}C NMR (101 MHz, DMSO) δ 165.74, 158.72, 137.22, 132.77, 131.62, 128.37, 66.82, 56.83, 49.93, 49.16.

2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)(trityl)amino)ethan-1-ol (**31**)

The compound was obtained from **29** (0.05 g, 0.19 mmol) as described for compound **30**. Purification by flash chromatography (60/40 heptane/EtOAc) to give 0.04 g (48%) of the desired product as a red solid. $R_f = 0.53$ (60/40 heptane/EtOAc); ^1H NMR (600 MHz, CDCl_3) δ 10.22 (s, 1H), 8.65 (d, $J = 8.2$ Hz, 2H), 7.86 (d, $J = 8.1$ Hz, 2H), 7.69 (d, $J = 7.9$ Hz, 6H), 7.33 (t, $J = 7.7$ Hz, 6H), 7.22 (t, $J = 7.3$ Hz, 3H), 3.76 (s, 2H), 3.29 (t, $J = 6.8$ Hz, 2H), 2.56 (d, $J = 7.0$ Hz, 2H), 1.63 (s, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 166.40, 157.73, 147.86, 143.28, 130.35, 129.37, 128.55, 128.49, 126.38, 79.05, 61.76, 57.79, 57.12.

2-((4-(1,2,4,5-Tetrazin-3-yl)benzyl)(trityl)amino)ethyl 4-nitrobenzenesulfonate (**9b**)

The compound was obtained from **31** (0.04 g, 0.08 mmol) as described for compound **3a**. Purification by flash chromatography (90/10 heptane/EtOAc) to give 0.04 g (48%) of the desired product as a red solid. $R_f = 0.25$ (80/20 heptane/EtOAc); ^1H NMR (400 MHz, CDCl_3) δ 10.19 (s, 1H), 8.46 (d, $J = 8.3$ Hz, 2H), 8.08 (d, $J = 8.8$ Hz, 2H), 7.69 (d, $J = 8.8$ Hz, 2H), 7.64 (d, $J = 8.2$ Hz, 2H), 7.61–7.43 (m, 6H), 7.23 (t, $J = 7.6$ Hz, 6H), 7.16–7.09 (m, 3H), 4.32–3.39 (m, 4H), 2.57 (t, $J = 7.2$ Hz, 2H); ^{13}C NMR (101 MHz, CDCl_3) δ 166.20, 157.92, 150.45, 146.36, 142.60, 141.64, 130.79, 129.11, 128.98, 128.91, 128.50, 127.98, 126.69, 124.19, 79.05, 57.38.

3.1.16. 2-Fluoroethyl 6-(4-(pyridin-2-yl)phenyl)nicotinate (**33**)

6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinic acid (**32**)

6-Cyanonicotinic acid (1.0 g, 6.75 mmol), 2-cyanopyridine (5.6 g, 45.01 mmol), and sulfur (0.43 g, 1.69 mmol) were suspended in EtOH (10 mL), followed by the addition of hydrazine hydrate (4.93 mL, 101.26 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature, and the formed precipitate was removed by filtration. Water (20 mL) and a solution of NaNO_2 (9.31 g, 135.32 mmol) in 50 mL water were added, and the mixture was cautiously acidified to pH 2–3 by addition of AcOH. The mixture was extracted with CH_2Cl_2 , and the combined organic layer was washed with water and brine, dried over MgSO_4 , and concentrated. The residue was purified by flash column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5 + 0.1% AcOH) to afford 0.52 g (27%) of the desired compound as a pink solid. $R_f = 0.21$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5 + 0.1% AcOH); ^1H NMR (400 MHz, DMSO) δ 9.37 (d, $J = 2.1$ Hz, 1H), 8.96 (q, $J = 1.6$ Hz, 1H), 8.71 (d, $J = 8.1$ Hz, 1H), 8.64 (d, $J = 7.9$ Hz, 1H), 8.58 (dd, $J = 8.1, 2.1$ Hz, 1H), 8.18 (td, $J = 7.8, 1.8$ Hz, 1H), 7.95–7.67 (m, 1H); ^{13}C NMR (101 MHz, DMSO) δ 165.74, 163.21, 162.97, 153.13, 151.01, 150.70, 149.96, 138.66, 137.86, 128.69, 126.81, 124.53, 124.

2-Fluoroethyl 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinate (**33**)

The compound was obtained from 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinic acid (0.05 g, 0.18 mmol) as described for compound **3** to give 0.55 g (95%) of the desired product as a pink solid. $R_f = 0.41$ (heptane/EtOAc 20/80); ^1H NMR (400 MHz, CDCl_3) δ 9.56 (dd, $J = 2.2, 0.9$ Hz, 1H), 9.00 (ddd, $J = 4.8, 1.8, 0.9$ Hz, 1H), 8.85 (dd, $J = 8.2, 0.9$ Hz, 1H), 8.77 (dt, $J = 7.9, 1.1$ Hz, 1H), 8.02 (td, $J = 7.8, 1.8$ Hz, 1H), 7.60 (ddd, $J = 7.7, 4.7, 1.2$ Hz, 1H), 4.88–4.79 (m, 1H), 4.72 (td, $J = 5.9, 3.6$ Hz, 2H), 4.64 (dd, $J = 5.0, 3.0$ Hz, 1H); ^{13}C NMR (101 MHz, CDCl_3) δ 164.34, 163.84, 163.50, 151.97, 149.86, 137.55, 127.81, 124.78, 123.95, 81.04 (d, $J = 171.5$ Hz), 64.62 (d, $J = 20.3$ Hz).

3.1.17. 2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl

6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinate (**33a**)

2-Hydroxyethyl 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinate (**34**)

The compound was obtained from **32** (0.04 g, 0.14 mmol) as described for compound **11**. The compound was used as a crude due to low solubility (0.035 g, 76%). $R_f = 0.31$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 90/10); ^1H NMR (400 MHz, CDCl_3) δ 9.47 (dd, $J = 2.1, 0.9$ Hz, 1H), 9.01–8.90 (m, 1H), 8.86–8.74 (m, 1H), 8.70 (dd, $J = 7.9, 1.1$ Hz, 1H), 8.55 (dd, $J = 8.2, 2.1$ Hz,

1H), 7.96 (ddt, $J = 10.4, 7.7, 2.2$ Hz, 1H), 7.62–7.48 (m, 1H), 4.76–4.45 (m, 2H), 4.26–3.68 (m, 2H), 3.24 (s, 1H).

2-(((4-Nitrophenyl)sulfonyl)oxy)ethyl 6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)nicotinate (**33a**)

The compound was obtained from **34** (0.035 g, 0.11 mmol) as reported for compound **3a**. Purification by flash chromatography (CH₂Cl₂/MeOH 95/5) afforded 0.035 g (64%) of the desired compound as a red solid. $R_f = 0.35$ (CH₂Cl₂/MeOH 95/5); ¹H NMR (400 MHz, DMSO) δ 9.25 (d, $J = 2.2$ Hz, 1H), 8.76 (d, $J = 8.4$ Hz, 2H), 8.66 (d, $J = 7.8$ Hz, 1H), 8.50 (dd, $J = 8.2, 2.2$ Hz, 1H), 8.40 (d, $J = 8.9$ Hz, 2H), 8.24 (d, $J = 8.9$ Hz, 2H), 8.22–8.17 (m, 1H), 7.87–7.68 (m, 1H), 4.63 (s, 4H); ¹³C NMR (101 MHz, DMSO) δ 164.1, 163.71, 163.51, 154.88, 154.14, 151.23, 150.39, 141.06, 139.06, 138.39, 129.87, 127.37, 125.42, 125.08, 124.59, 123.78, 70.42, 63.28.

3.1.18. 5-((2-Fluoroethoxy)methyl)-2-(4-(pyridin-2-yl)phenyl)pyridine (**38**)

5-(Bromomethyl)picolinonitrile (**35**)

To a solution of 5-methylpicolinonitrile (7.00 g, 59.25 mmol) and N-bromo succinimide (13.71 g, 77.03 mmol) in CH₃CN (100 mL) was added AIBN (3.89 g, 23.70 mmol). The resulting solution was refluxed for 12 h. The reaction was cooled down, and EtOAc (200 mL) was added. The organic layer was washed with water (2 × 100 mL) and brine (2 × 100 mL), dried over anhydrous Na₂SO₄, filtered, and concentrated under reduced pressure. Purification by flash chromatography (n-heptane/EtOAc 90/10) afforded 7.1 g (61%) of **35** as a white solid. $R_f = 0.29$ (n-heptane/EtOAc 80/20); ¹H NMR (400 MHz, CDCl₃) δ 8.72 (d, $J = 2.2$ Hz, 1H), 7.87 (dd, $J = 8.1, 2.3$ Hz, 1H), 7.69 (dd, $J = 8.1, 0.9$ Hz, 1H), 4.49 (s, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 151.19, 137.50, 137.45, 133.40, 128.36, 116.84, 27.87.

5-((2-Hydroxyethoxy)methyl)picolinonitrile (**36**)

NaH (60% weight, 0.22 g, 5.58 mmol) was suspended in dry THF (10 mL), and ethylene glycol (2.83 mL, 50.75 mmol) was added dropwise under argon at 0 °C. The solution was left for 30 min at 0 °C before dropwise addition of 5-(bromomethyl)picolinonitrile (1.00 g, 5.87 mmol) in dry THF (10 mL) under argon at 0 °C. The reaction was stirred to room temperature for 10 min and refluxed for 3 h. The reaction was cooled to room temperature and quenched by adding EtOAc (40 mL), and the crude was washed with NH₄Cl (sat, 50 mL × 2) and water (50 mL). The organic phase was dried over Na₂SO₄ and concentrated under reduced pressure. Purification by flash chromatography (DCM/MeOH 98/2) afforded 0.55 g (61%) of the desired product as a yellow oil. $R_f = 0.31$ (heptane/EtOAc 30/70); ¹H NMR (400 MHz, CDCl₃) δ 8.60 (d, $J = 2.2$ Hz, 1H), 7.82 (dd, $J = 8.1, 2.2$ Hz, 1H), 7.95–7.32 (m, 1H), 4.61 (s, 2H), 3.74 (dd, $J = 5.4, 3.8$ Hz, 2H), 3.61 (dd, $J = 5.4, 3.8$ Hz, 2H); ¹³C NMR (101 MHz, CDCl₃) δ 149.94, 138.24, 135.89, 132.41, 128.30, 117.20, 72.47, 69.77, 61.51.

2-((6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methoxy)ethan-1-ol (**37**)

Compound **36** (0.55 g, 3.08 mmol), 2-cyanopyridine (1.6 g, 15.43 mmol), and sulfur (0.2 g, 0.77 mmol) were suspended in EtOH (5 mL), followed by the addition of hydrazine hydrate (2.26 mL, 43.3 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature, and the formed precipitate was removed by filtration. Water (20 mL) and a solution of NaNO₂ (4.26 g, 61.73 mmol) in 30 mL water were added, and the mixture was cautiously acidified to pH 2 by addition of AcOH. The mixture was extracted with CH₂Cl₂, and the combined organic layer was washed with water and brine, dried over MgSO₄, and concentrated. The residue was purified by flash column chromatography (CH₂Cl₂/MeOH 95/5) to afford 0.4 g (42%) of the desired compound as a pink solid. $R_f = 0.21$ (CH₂Cl₂/MeOH 95/5); ¹H NMR (400 MHz, CDCl₃) δ 9.03–8.94 (m, 1H), 8.90 (d, $J = 2.1$ Hz, 1H), 8.79–8.65 (m, 2H), 8.00 (ddd, $J = 7.9, 5.5, 2.1$ Hz, 3H), 7.57 (ddd, $J = 7.6, 4.7, 1.2$ Hz, 1H), 4.74 (s, 2H), 3.88–3.78 (m, 2H), 3.70 (dd, $J = 5.3, 3.9$ Hz, 2H), 2.76 (s, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 163.79, 163.69, 150.99, 150.03, 149.30, 137.50, 137.21, 136.47, 126.59, 124.50, 124.25, 72.24, 70.26, 61.80.

3-(5-((2-Fluoroethoxy)methyl)pyridin-2-yl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**38**)

The compound was obtained from 2-((6-(6-(pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methoxy)ethan-1-ol (0.1 g, 0.33 mmol) dissolved in 5 mL dry THF and PBSF (0.20 mg,

0.66 mmol), and DIPEA (0.34 mL, 1.93 mmol) was added. Et₃N·3HF (0.11 mL, 0.66 mmol) was dissolved in 2 mL THF and added dropwise. The reaction was left at room temperature for 12 h. The reaction was diluted with 20 mL CH₂Cl₂ and washed with NH₄Cl (sat). The aqueous phase was extracted with CH₂Cl₂ (2 × 10 mL), and the combined organic layers were dried over MgSO₄ and concentrated under reduced pressure. Purification by flash chromatography (CH₂Cl₂/MeOH 95/5) afforded 0.075 g (75%) of the desired product as a pink solid. R_f = 0.45 (CH₂Cl₂/MeOH 95/5); ¹H NMR (400 MHz, CDCl₃) δ 9.05–8.80 (m, 2H), 8.78–8.58 (m, 2H), 8.21–7.86 (m, 2H), 7.51 (ddd, *J* = 7.6, 4.7, 1.2 Hz, 1H), 4.71 (s, 2H), 4.65–4.58 (m, 1H), 4.57–4.42 (m, 1H), 3.97–3.78 (m, 1H), 3.77–3.68 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 163.82, 163.73, 150.98, 150.03, 149.97, 149.36, 137.50, 137.03, 136.43, 126.60, 124.48, 124.26, 82.98 (d, *J* = 169.5 Hz), 70.35, 70.00 (d, *J* = 19.6 Hz).

3.1.19. 2-((6-(4-(Pyridin-2-yl)phenyl)pyridin-3-yl)methoxy)ethyl 4-methylbenzene sulfonate (**38a**)

The compound was obtained from **37** (0.05 g, 0.17 mmol) as reported for compound **3a**. Purification by flash chromatography (CH₂Cl₂/MeOH 98/2) afforded 0.07 g (84%) of the desired compound as a red oil. R_f = 0.48 (CH₂Cl₂/MeOH 90/10); ¹H NMR (600 MHz, CDCl₃) δ 9.02 (dt, *J* = 4.5, 1.4 Hz, 1H), 8.86 (d, *J* = 2.1 Hz, 1H), 8.80–8.63 (m, 2H), 8.41–8.33 (m, 1H), 8.17–8.11 (m, 1H), 8.04 (td, *J* = 7.7, 1.8 Hz, 1H), 7.93 (dd, *J* = 8.0, 2.2 Hz, 1H), 7.61 (ddd, *J* = 7.6, 4.7, 1.1 Hz, 1H), 4.69 (s, 2H), 4.44–4.38 (m, 2H), 3.90–3.82 (m, 2H); ¹³C NMR (151 MHz, CDCl₃) δ 163.90, 163.68, 151.09, 150.76, 150.11, 150.00, 149.76, 141.81, 137.50, 136.40, 136.21, 129.25, 126.62, 124.58, 124.41, 124.22, 70.44, 70.15, 68.20.

3.1.20. 5-(2-Fluoroethoxy)-2-(4-(pyridin-2-yl)phenyl)pyridine (**41**)

5-(2-Fluoroethoxy)picolinonitrile (**39**)

The compound was obtained from 5-hydroxypicolinonitrile (0.5 g, 4.16 mmol) as described for compound **20** to give 0.56 g (81%) of the desired product as a pink solid. R_f = 0.34 (heptane/EtOAc 70/30); ¹H NMR (400 MHz, CDCl₃) δ 8.34 (d, *J* = 2.9 Hz, 1H), 7.59 (d, *J* = 8.6 Hz, 1H), 7.27–7.20 (m, 1H), 4.83–4.76 (m, 1H), 4.71–4.64 (m, 1H), 4.35–4.28 (m, 1H), 4.28–4.21 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 155.04, 138.50, 127.74, 123.96, 118.83, 115.55, 79.47 (d, *J* = 172.4 Hz), 66.05 (d, *J* = 20.4 Hz).

3-(5-(2-Fluoroethoxy)pyridin-2-yl)-6-(pyridin-2-yl)-1,2,4,5-tetrazine (**41**)

The compound was obtained from **39** (0.55 g, 3.31 mmol) as described for compound **37**, followed by purification by preparative HPLC to give 0.04 g (4%) of the desired product as a pink solid. R_f = 0.32 (CH₂Cl₂/MeOH 97/3); ¹H NMR (400 MHz, CDCl₃) δ 8.94 (dt, *J* = 4.7, 1.4 Hz, 1H), 8.70 (d, *J* = 8.5 Hz, 2H), 8.65 (d, *J* = 2.9 Hz, 1H), 8.00 (td, *J* = 7.8, 1.7 Hz, 1H), 7.57 (ddd, *J* = 7.7, 4.8, 1.2 Hz, 1H), 7.46 (dd, *J* = 8.8, 2.9 Hz, 1H), 4.88–4.81 (m, 1H), 4.76–4.69 (m, 1H), 4.44–4.38 (m, 1H), 4.38–4.25 (m, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 163.26, 163.21, 157.38, 150.46, 149.69, 142.33, 139.43, 138.19, 126.84, 125.92, 124.58, 121.99, 81.39 (d, *J* = 172.7 Hz), 67.99 (d, *J* = 20.4 Hz); HPLC-MS [M+H]⁺ *m/z* calc. for [C₁₄H₁₂FN₆O]⁺: 299.10; found: 299.12.

3.1.21. 2-((6-(4-(Pyridin-2-yl)phenyl)pyridin-3-yl)oxy)ethyl 4-nitrobenzenesulfonate (**41a**)

5-(2-Hydroxyethoxy)picolinonitrile (**40**)

The compound was obtained from 5-hydroxypicolinonitrile (1.4 g, 11.65 mmol) as described for compound **21**. Purification by flash chromatography (n-heptane/EtOAc 40/60) afforded 1.45 g (76%) of the desired product as a white solid. R_f = 0.21 (heptane/EtOAc 40/60); ¹H NMR (400 MHz, CDCl₃) δ 8.32 (d, *J* = 2.9 Hz, 1H), 7.59 (d, *J* = 8.6 Hz, 1H), 7.42–6.71 (m, 1H), 4.66–4.05 (m, 2H), 3.97 (dd, *J* = 5.1, 3.9 Hz, 2H), 2.27 (s, 1H); ¹³C NMR (101 MHz, CDCl₃) δ 157.27, 140.42, 129.65, 125.41, 120.58, 117.39, 70.21, 60.86.

2-((6-(6-(Pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)oxy)ethan-1-ol (**42**)

The compound was obtained from **40** (0.5 g, 3.04 mmol) as described for compound **37**. Purification by flash chromatography (CH₂Cl₂/MeOH 97/3) afforded 0.27 g (30%) of the desired product as a pink solid. R_f = 0.25 (CH₂Cl₂/MeOH 95/5); ¹H NMR (600 MHz,

DMSO) δ 8.93 (ddd, $J = 4.7, 1.8, 0.9$ Hz, 1H), 8.65 (dd, $J = 2.9, 0.6$ Hz, 1H), 8.62–8.49 (m, 2H), 8.16 (td, $J = 7.7, 1.8$ Hz, 1H), 7.82–7.56 (m, 2H), 5.00 (s, 1H), 4.50–3.97 (m, 2H), 3.81 (td, $J = 5.4, 4.3$ Hz, 2H); ^{13}C NMR (151 MHz, DMSO) δ 163.49, 163.30, 157.81, 151.05, 150.71, 142.37, 139.97, 138.25, 127.00, 126.05, 124.59, 121.95, 70.96, 59.88.

2-((6-(4-(Pyridin-2-yl)phenyl)pyridin-3-yl)oxy)ethyl 4-nitrobenzenesulfonate (**41a**)

The compound was obtained from **42** (0.05 g, 0.17 mmol) as reported for compound **3a**. Purification by flash chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 98/2) afforded 0.03 g (37%) of the desired compound as a red solid (mixture of conformers 70/30). $R_f = 0.61$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 90/10); ^1H NMR (600 MHz, DMSO) δ 8.94 (ddd, $J = 4.7, 1.8, 0.9$ Hz, 1H), 8.70 (d, $J = 2.9$ Hz, 0.3H), 8.65 (d, $J = 8.5$ Hz, 0.3H), 8.60 (d, $J = 7.8$ Hz, 1H), 8.56 (d, $J = 8.8$ Hz, 0.7H), 8.51 (d, $J = 2.9$ Hz, 0.7H), 8.47–8.43 (m, 1.4H), 8.27–8.22 (m, 1.4H), 8.21–8.18 (m, 0.6H), 8.16 (td, $J = 7.8, 1.8$ Hz, 1H), 7.90–7.81 (m, 0.6H), 7.78 (dd, $J = 8.8, 2.9$ Hz, 0.3H), 7.73 (ddd, $J = 7.6, 4.7, 1.2$ Hz, 1H), 7.64 (dd, $J = 8.8, 3.0$ Hz, 0.7H), 4.81–4.71 (m, 0.6H), 4.68–4.59 (m, 1.4H), 4.57–4.54 (m, 0.6H), 4.51–4.43 (m, 1.4H); ^{13}C NMR (151 MHz, DMSO) δ 163.57, 163.53, 163.26, 163.21, 156.74, 156.60, 154.89, 151.16, 151.08, 150.66, 147.70, 143.22, 142.95, 140.95, 139.86, 139.77, 138.28, 129.93, 127.38, 127.08, 127.05, 126.08, 125.89, 125.43, 124.63, 123.78, 122.36, 122.12, 74.80, 70.58, 67.80, 66.45.

3.1.22. 2,2'-(((6-(6-(5-(2-Fluoroethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azanediyl)diacetic Acid (**45**)

Di-*tert*-butyl 2,2'-(((6-cyanopyridin-3-yl)methyl)azanediyl)diacetate (**43**)

To a solution of 5-(bromomethyl)picolinonitrile (1.5 g, 7.61 mmol) in CH_3CN (50 mL) was added K_2CO_3 (3.15 g, 22.84 mmol) and di-*tert*-butyl iminodiacetate (1.96 g, 7.99 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 \times 40 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 2.7 g (98%) of the desired compound as a white solid. $R_f = 0.33$ (*n*-heptane/EtOAc 80/20); ^1H NMR (400 MHz, CDCl_3) δ 8.61 (dd, $J = 2.1, 0.9$ Hz, 1H), 7.96 (dd, $J = 8.0, 2.1$ Hz, 1H), 7.60 (dd, $J = 7.9, 0.9$ Hz, 1H), 3.93 (s, 2H), 3.33 (s, 4H), 1.40 (s, 18H); ^{13}C NMR (101 MHz, CDCl_3) δ 170.09, 151.41, 138.96, 137.51, 132.77, 128.27, 117.35, 81.52, 55.41, 54.57, 28.16.

Di-*tert*-butyl 2,2'-(((6-(6-(5-(2-fluoroethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azanediyl)diacetate (**44**)

Compound **43** (0.2 g, 0.55 mmol), compound **39** (0.46 g, 2.77 mmol), and sulfur (0.36 g, 0.14 mmol) were suspended in EtOH (5 mL), followed by the addition of hydrazine hydrate (0.40 mL, 8.31 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature and the formed precipitate was removed by filtration. Water (20 mL) and a solution of NaNO_2 (0.76 g, 11.07 mmol) in 10 mL water were added, and the mixture was cautiously acidified to pH 2–3 by addition of AcOH. The mixture was extracted with CH_2Cl_2 , and the combined organic layer was washed with water and brine, dried over MgSO_4 , and concentrated. The residue was purified by flash column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5) and crystallized from MeOH to afford 0.07 g (23%) of the desired compound as a pink solid. $R_f = 0.36$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5); ^1H NMR (600 MHz, DMSO) δ 8.86 (d, $J = 2.1$ Hz, 1H), 8.68 (dd, $J = 7.9, 3.1$ Hz, 1H), 8.62 (d, $J = 8.8$ Hz, 1H), 8.58 (d, $J = 8.0$ Hz, 1H), 8.11 (dd, $J = 8.1, 2.1$ Hz, 1H), 7.76 (dd, $J = 8.8, 3.0$ Hz, 1H), 4.97–4.86 (m, 1H), 4.81 (dd, $J = 4.8, 2.8$ Hz, 1H), 4.54 (dd, $J = 4.8, 2.7$ Hz, 1H), 4.51–4.43 (m, 1H), 4.01 (s, 2H), 3.45 (s, 4H), 1.43 (s, 18H); ^{13}C NMR (151 MHz, DMSO) δ 170.35, 163.47, 163.24, 157.21, 151.20, 149.63, 142.89, 139.86, 138.19, 138.11, 126.00, 124.30, 82.43 (d, $J = 166.8$ Hz), 80.97, 68.39 (d, $J = 18.9$ Hz), 55.57, 54.87, 28.29.

2,2'-(((6-(6-(5-(2-Fluoroethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azane-diyl)diacetic acid (**45**)

To a solution of **44** (0.006 g, 0.83 mmol) in 5 mL of CH_2Cl_2 was added 2 mL of TFA. The mixture was stirred at room temperature for 4 h. The solvent was then removed under

reduced pressure. Purification by preparative HPLC afforded 0.35 g (58%) of the desired compound (TFA salt) as a red solid. ^1H NMR (600 MHz, DMSO) δ 8.89 (d, $J = 2.1$ Hz, 1H), 8.68 (d, $J = 2.9$ Hz, 1H), 8.62 (d, $J = 8.8$ Hz, 1H), 8.57 (dd, $J = 8.0, 0.8$ Hz, 1H), 8.14 (dd, $J = 8.1, 2.1$ Hz, 1H), 7.76 (dd, $J = 8.8, 3.0$ Hz, 1H), 4.91–4.86 (m, 1H), 4.83–4.79 (m, 1H), 4.57–4.52 (m, 1H), 4.52–4.47 (m, 1H), 4.06 (s, 2H), 3.53 (s, 4H); ^{13}C NMR (151 MHz, DMSO) δ 172.62, 163.47, 163.23, 157.22, 151.33, 149.58, 142.89, 139.86, 138.28, 126.00, 124.27, 122.16, 82.43 (d, $J = 166.8$ Hz), 68.39 (d, $J = 18.8$ Hz), 55.00, 54.49; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{19}\text{H}_{19}\text{FN}_7\text{O}_5]^+$: 444.14; found: 444.13.

3.1.23. Di-*tert*-butyl 2,2'-(((6-(6-(5-(2-(((4-nitrophenyl)sulfonyl)oxy)ethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azanediyl)diacetate (**45a**)

Di-*tert*-butyl 2,2'-(((6-(6-(5-(2-hydroxyethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azanediyl)diacetate (**46**)

Compound **43** (1.05 g, 2.92 mmol), compound **40** (0.12 g, 0.73 mmol), and sulfur (0.05 g, 0.18 mmol) were suspended in EtOH (3 mL), followed by the addition of hydrazine hydrate (0.53 mL, 10.96 mmol). The reaction was heated to 90 °C for 2 h. The mixture was cooled to room temperature and the formed precipitate was removed by filtration. Water (10 mL) and a solution of NaNO_2 (1.0 g, 14.62 mmol) in 10 mL water were added, and the mixture was cautiously acidified to pH 2 by addition of AcOH. The mixture was extracted with CH_2Cl_2 , and the combined organic layer was washed with water and brine, dried over MgSO_4 , and concentrated. The residue was purified by flash column chromatography ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5) to give 0.11 g (27%) of the desired compound as a pink solid. $R_f = 0.33$ ($\text{CH}_2\text{Cl}_2/\text{MeOH}$ 95/5); ^1H NMR (400 MHz, MeOD) δ 8.88 (d, $J = 2.0$ Hz, 1H), 8.81–8.69 (m, 2H), 8.56 (d, $J = 2.9$ Hz, 1H), 8.22 (dd, $J = 8.1, 2.1$ Hz, 1H), 7.71 (dd, $J = 8.9, 2.9$ Hz, 1H), 4.45–4.25 (m, 2H), 4.10 (s, 2H), 4.04–3.93 (m, 2H), 3.50 (s, 4H), 3.33 (t, $J = 1.6$ Hz, 1H), 1.50 (s, 18H); ^{13}C NMR (101 MHz, MeOD) δ 170.55, 163.15, 163.04, 158.24, 150.67, 148.89, 141.66, 139.29, 138.58, 138.49, 125.57, 123.75, 121.49, 81.08, 70.31, 59.99, 55.12, 54.74, 27.04.

Di-*tert*-butyl 2,2'-(((6-(6-(5-(2-(((4-nitrophenyl)sulfonyl)oxy)ethoxy)pyridin-2-yl)-1,2,4,5-tetrazin-3-yl)pyridin-3-yl)methyl)azanediyl)diacetate (**45a**)

The compound was obtained from **46** (0.05 g, 0.09 mmol) as reported for compound **3a**. Purification by flash chromatography (heptane/EtOAc 40/60) afforded 0.045 g (67%) of the desired compound as a red solid. $R_f = 0.26$ (heptane/EtOAc 30/70); ^1H NMR (600 MHz, CDCl_3) δ 8.82 (d, $J = 2.1$ Hz, 1H), 8.70–8.59 (m, 2H), 8.48 (d, $J = 2.9$ Hz, 1H), 8.35 (d, $J = 8.8$ Hz, 2H), 8.15–7.98 (m, 3H), 7.32 (d, $J = 2.9$ Hz, 1H), 4.76–4.47 (m, 2H), 4.41–4.31 (m, 2H), 4.01 (s, 2H), 3.40 (s, 4H), 1.41 (s, 18H); ^{13}C NMR (151 MHz, CDCl_3) δ 169.22, 162.64, 162.15, 155.32, 150.32, 149.95, 148.26, 142.30, 140.49, 138.34, 137.23, 137.03, 128.33, 124.54, 123.55, 123.24, 120.47, 80.37, 67.51, 65.00, 54.31, 53.61, 27.18; HPLC-MS $[\text{M}+\text{H}]^+$ m/z calc. for $[\text{C}_{33}\text{H}_{39}\text{N}_8\text{O}_{10}\text{S}]^+$: 739.25; found: 739.26.

3.2. Radiochemistry

3.2.1. [^{18}F]Fluoride Production and General Methods

The [^{18}F]Fluoride was produced by a cyclotron CTI Siemens Eclipse, Rigshospitalet, Denmark, by irradiating [^{18}O]H $_2$ O via a (p,n) reaction. Automated synthesis was performed on a Scansys 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 \times 4.6 mm). Eluents: A, H $_2$ O 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 was at $\lambda = 254$ nm on a UVD 170U detector, and radioactivity was analyzed with a flow-through GM-tube-based radiodetector (Scansys).

3.2.2. 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 afterward. The [^{18}F] F^- was eluted from the QMA into a 4 mL v-shaped vial using 1 mL Bu_4NOMs (20 μmol , 6,8 mg) dissolved in MeOH. The eluate was dried at 100 $^\circ\text{C}$ for 5 min under N_2 flow. Compound **45a** was dissolved in 167 μL DMSO and then diluted with 833 μL *t*BuOH (1:5 ratio). The solution was added to the dried fluoride solution and allowed to react for 5 min at 100 $^\circ\text{C}$. The reaction was cooled to 50 $^\circ\text{C}$ with air before addition of 3 mL H_2O . This mixture was applied to a Sep-Pak C18 Plus 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 $^\circ\text{C}$. 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 was purified by semipreparative HPLC (Luna 5 μm C18(2) 100 \AA , 250 mm \times 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.

3.2.3. Tetrazine Core Reactivity Test

The reaction between [^{18}F]**45** and TCO-PNB was performed by mixing the formulated [^{18}F]**45** (200 μL) with 5 μL of the commercially available TCO-PNB 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 into the analytical HPLC instrument for analysis.

3.3. Blocking Assay and Ex Vivo Studies

3.3.1. Establishing Tumor Xenografts in Mice

All animal studies were approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries of Denmark (license no. 2016-15-0201-00920).

The human colon cancer cell line LS174T (ATCC, Manassas, VA, USA) was cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% *L*-glutamine, 1% sodium pyruvate, 1% nonessential amino acids, and 1% penicillin–streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). The cells were trypsinized and harvested for inoculation when they were in their exponential growth phase.

Subcutaneous tumors were established in the flank of five-week-old female nude BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France) by inoculation of $\sim 5 \times 10^6$ LS174T cells (in 100 μL sterile PBS).

The tumor volume was estimated from caliper measurements using the formula: $\text{volume} = \frac{1}{2} (\text{length} \times \text{width}^2)$.

3.3.2. Blocking Experiments

The blocking experiments were performed as previously described [17,32]. Briefly, tumor-bearing animals were grouped based on their tumor volume (~ 100 – 300 mm^3 , $n = 3/\text{group}$) and administered 100 $\mu\text{g}/100 \mu\text{L}$ of CC49-TCO per mouse ($\sim 7 \text{ TCO}/\text{mAb}$). The ability of nonradioactive Tzs to block the binding between [^{111}In]**47** and CC49-TCO was evaluated three days later. First, the animals were injected with the (nonradioactive) Tzs (39 nmol) that were chosen for in vivo evaluation. After a lag time of 1 h, [^{111}In]**47** ($\sim 5 \text{ MBq}$, 3.9 nmol) was administered, and after 22 h, the mice were euthanized. Tissues were resected and weighted, and the radioactivity was measured using a gamma counter (Wizard2, Perkin Elmer). Data were corrected for decay, tissue weight, and injected amount of radioactivity. The setup also included a control group of mice receiving the precursor of [^{111}In]**47** instead of a test compound 1 h prior to [^{111}In]**47** (positive control), as well as a

group exclusively receiving [^{111}In]47. The tumor uptake of the different evaluated Tzs was normalized to the tumor uptake of the latter to determine the blocking effect.

3.3.3. Pretargeted Imaging

LS174T xenografts were established in mice as previously described. When tumors reached a size of $\sim 100\text{ mm}^3$, animals were divided into two groups based on their tumor volume ($n = 5/\text{group}$), and injected iv with $50\text{ }\mu\text{g}$ in $100\text{ }\mu\text{L}$ of either CC49-TCO or CC49. After 72 h the animals were administered intravenously with [^{18}F]45 ($4.74 \pm 1.39\text{ MBq}$ in $100\text{ }\mu\text{L}$ of PBS) and scanned using PET/CT (Inveon, Siemens Medical Solutions, USA) 1 h later, using a PET acquisition time of 5 min, an energy window of 350–650 KeV, and a time resolution of 6 ns; followed by a continuous 360 projection/360° CT scan, acquired with an X-ray tube voltage of 65 kV, a tube anode current of $500\text{ }\mu\text{A}$, and an exposure time per projection of 400 ms. Mice were anesthetized by breathing 3% sevoflurane (5% for induction) mixed in 35% O_2 in N_2 , and during scans the body temperature of the mice was kept stable using a heating pad.

Signograms from PET scans were reconstructed using a three-dimensional maximum a posteriori algorithm with correction for scatter and attenuation. The mean percentage of injected dose per grams (%ID/mL) was determined by manually creating regions of interest (ROI) on coregistered PET/CT images (Inveon Research Workplace software, Siemens Medical Solutions, Malvern, PA, USA).

GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis, and an unpaired *t*-test with Welch's correction was used to compare the tumor uptake in the two groups. Results were considered significant when $p < 0.05$.

4. Conclusions

In this study, we reported the development of the first bispyridyl Tz directly labeled with fluorine-18. The [^{18}F]45 was obtained with sufficient yield, purity, and molar activity for in vivo evaluation. This imaging agent had comparable performances and target-to-background ratios compared to previously reported successful Tz tracers. Further evaluation studies are needed in order to optimize its potential for pretargeted imaging. The developed [^{18}F]45 is of special interest in pretargeted imaging, as it may be used—as a bispyridyl-based pretargeted imaging agent—to quantify drug release based on 'click-to-release' approaches. Bispyridyls have recently been shown to near-quantitatively release drugs from iTCOs within $<10\text{ min}$ [41]. This is so far the fastest release demonstrated. The ability to quantify in vivo drug release is essential to precisely fine-tune dosing—one of the most important factors influencing the effectiveness of a treatment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ph15020245/s1>. The supplementary material is NMR spectra of all the compounds and radio-HPLC of the final compounds.

Author Contributions: R.G.-V. and J.T.J. contributed equally to the work. Organic synthesis was performed by U.M.B., and subsequent radiolabeling experiments were performed by R.G.-V. and K.E.B. In vivo studies and PET experiments were performed by J.T.J., L.H. and V.S. The study was conceptionally designed by U.M.B., A.K. and M.M.H. with the help of all authors. The manuscript was written by U.M.B. and M.M.H. with contributions from all authors. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: All animal experiments in this study were approved by national animal welfare committees in Denmark, and the experiments were performed in accordance with European guidelines. All animal experiments were performed under a protocol approved by the Animal Research Committee of the Danish Ministry of Environment and Food (license no. 2016-15-0201-00920) and the Animal Ethics Committee of the University of Copenhagen, and were in compliance with the guidelines in Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data is contained within the article and Supplementary Materials.

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Abbreviations

NIR	Near-infrared
TPSA	Topological polar surface area
TCO-PNP	Para-nitrophenol trans-cyclooctene
EOS	End of synthesis
Bu ₄ NOMs	Tetrabutylammonium mesylate

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