Journal of Medicinal Chemistry

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Article

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

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Cite This: J. Med. Chem. 2021, 64, 15297−15312

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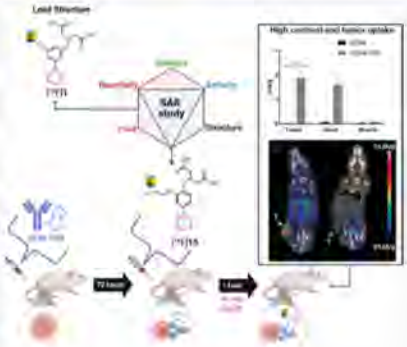
ABSTRACT: Pretargeted imaging of nanomedicines have attracted considerable interest because it has the potential to increase imaging contrast while reducing radiation burden to healthy tissue. Currently, the tetrazine ligation is the fastest bioorthogonal reaction for this strategy and, consequently, the state-of-art choice for *in vivo* chemistry. We have recently identified key properties for tetrazines in pretargeting. We have also developed a method to ^{18}F-label reactive tetrazines using an aliphatic nucleophilic substitution strategy. Here, we combined this knowledge and developed an ^{18}F-labeled tetrazine for pretargeted imaging. In order to develop this ligand, a small SAR study was performed. The most promising compound was selected for labeling and subsequent positron-emission-tomography *in vivo* imaging. Radiolabeling was achieved in satisfactory yields, molar activities, and high radiochemical purities. [^{18}F]**15** displayed favorable pharmacokinetics and remarkable target-to-background ratios—as early as 1 h post injection. We believe that this agent could be a promising candidate for translation into clinical studies.

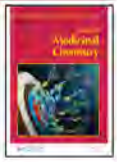


INTRODUCTION

Nanomedicines such as monoclonal antibodies or other nanoparticles have received increased interest, for example, 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 of up to 30−50% injected activity per kg (IA/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 need 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, with respect to the highest effectiveness with tolerable side-effects (theranostic concept) (Figure 1A).[6−8] Nanomedicines usually possess slow pharmacokinetics, that is, slow target accumulation and slow excretion. These processes can take days to weeks.[9,10] For targeted radionuclide approaches, this presents a challenge because high radiation doses are then delivered to healthy tissues, which limits and often prohibits the clinical application of these compounds.[9,10]

Pretargeting offers an intriguing alternative, which circumvents the dose limitations that conventional nanomedicine-based radionuclide therapies possess.[11,12] Pretargeted strategies allow the labeling of 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 within minutes—while unreacted tags are excreted rapidly. Thus, good target-to-background ratios can be obtained after minutes (Figure 1B). Consequently, the 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

Received: July 26, 2021
Published: October 15, 2021



https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312

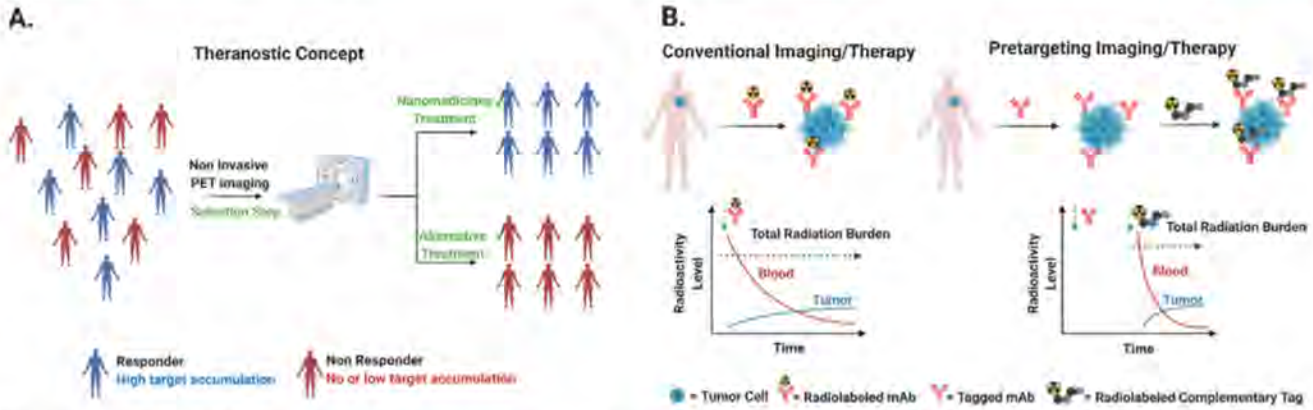


Figure 1. Application of nanomedicines for precision medicine. (A) Theranostic concept: with imaging, possible responders to a certain therapy can be identified. If identified, the therapy can be initiated. Otherwise, an alternative therapeutic strategy should be used. (B) Comparison between conventional 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.

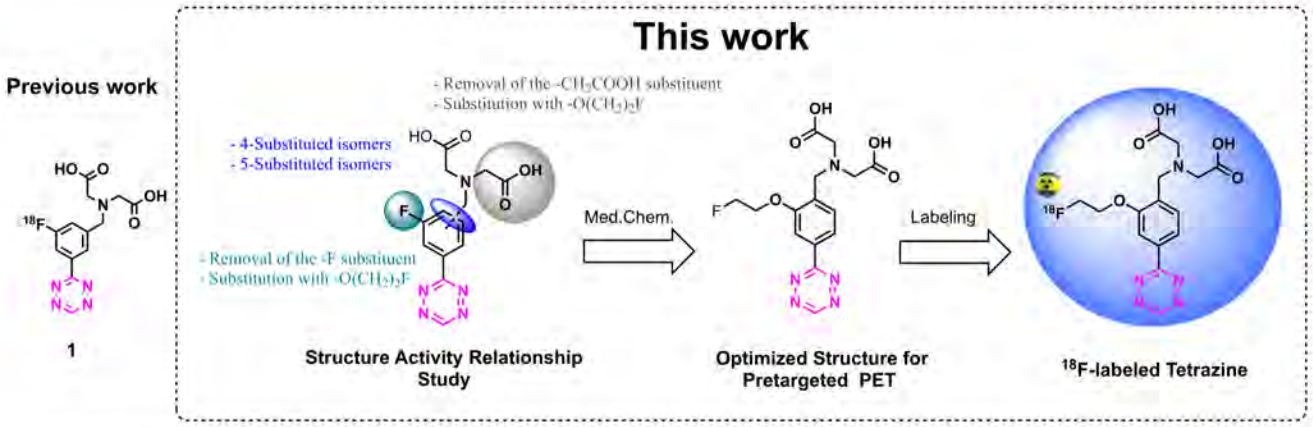


Figure 2. Design strategy to develop an aliphatic ^{18}F-Tz suitable for *in vivo* chemistry. The starting point for our structure activity relationship study was based on compound **1**—a recently reported successful pretargeted imaging agent. Our aim was to develop an agent without the use of a Cu-mediated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

view, fluorine-18 (^{18}F) 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.

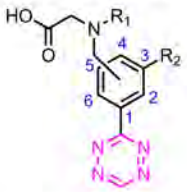
RESULT AND DISCUSSION

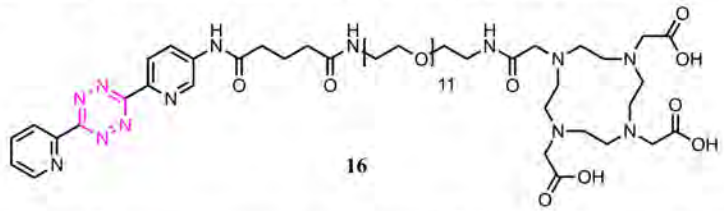
Design Strategy. Compound **1** was selected as the starting point for the design of new tracers. This compound was designed in accordance with our findings that high reactivity [>50.000 M^{-1} s^{-1} with TCO in Dulbecco's phosphate buffered saline (DPBS)] and low lipophilicity (cLog $D_{7.4}$ < −3) determine the *in vivo* ligation ability of Tzs for pretargeted strategies.[25] Indeed, [^{18}F]**1** showed good tumor-to-background ratios *in vivo*—already 1 h 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 permissible 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 from the aromatic ring, and (d) varying the substitution pattern of the phenyl ring influence the possibility of Tzs to be ligated *in vivo* (Figure 2). In this respect, we designed 15 compounds (Table 1).

Reference Compounds Synthesis. The key step in the synthesis of all reference compounds was the formation of the *H*-phenyl-Tz 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

https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312

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 Tz Derivatives





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tetrazine[a]	side chain position	R_1	R_2	Clog $D_{7.4}$[b]	TPSA[c]	rate constant (M^{-1} s^{-1})[d]	blocking effect[g]	% tumor uptake of [^{111}In]16 after blocking
1[e]	5	$-CH_2COOH$	$-F$	−6.93	129.40	82,000	90[h]	10[h]
2[e]	5	$-H$	$-F$	−2.89	100.89	78,000	95	5
3[e]	5	$-CH_2COOH$	$-H$	−6.97	129.40	62,000	95	5
4[e]	5	$-H$	$-H$	−3.03	100.89	55,000	96	4
5[e]	5	$-CH_2CH_2F$	$-F$	−2.67	92.10	88,000	76	24
6[e]	5	$-CH_2CH_2F$	$-H$	−2.73	92.10	62,000	75	25
7[e]	4	$-CH_2COOH$	$-F$	−6.91	129.40	76,000	98	2
8[e]	4	$-H$	$-F$	−2.98	100.89	76,000	98	2
9[e]	4	$-CH_2COOH$	$-H$	−6.98	129.40	62,000	97[i]	3[i]
10[e]	4	$-H$	$-H$	−3.03	100.89	60,000	86[i]	14[i]
11[e]	4	$-CH_2CH_2F$	$-F$	−2.77	92.10	74,000	72	28
12[e]	4	$-CH_2CH_2F$	$-H$	−2.73	92.10	55,000	69	31
13[e]	4	$-CH_2COOH$	$-OH$	−7.43	149.63	n.d.	99	1
14[e]	4	$-CH_2COOH$	$-OCH_3$	−7.24	138.63	68,000	99	1
15[e]	4	$-CH_2COOH$	$-OCH_2CH_2F$	−6.83	138.63	68,000	98	2
16[e]				−4.13[f]	358.03	74,000	99[f]	1[f]

[a]Notes: the compounds were obtained as trifluoroacetate salt. [b]Calculated distribution coefficients 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 DPBS at 37 °C (see experimental part 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 the $n = 3$ mice/group; detailed information can be found in the experimental part. [h]Blocking data from ref 26. [i]Blocking data from ref 25.

higher yields for H-phenyl-Tzs compared to those from "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 then reacts 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.*, with $NaNO_2$) to their corresponding Tzs. *N*-Benzyliminodiacetic acid derivatives **1**, **3**, **7**, and **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 Tz 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. Tz formation and deprotection resulted in the desired products as well. The reaction of *tert*-butyl glycine nitrile derivatives with 1-fluoro-2-iodoethane yielded the necessary intermediates to obtain **5**, **6**, **11**, and **12**, which were synthesized with a similar strategy to the one reported above (Scheme 1). Compounds **13**−**15** were obtained likewise (Scheme 2). Protection of the phenolic group of **45** and radical bromination followed by alkylation gave compound **49**.

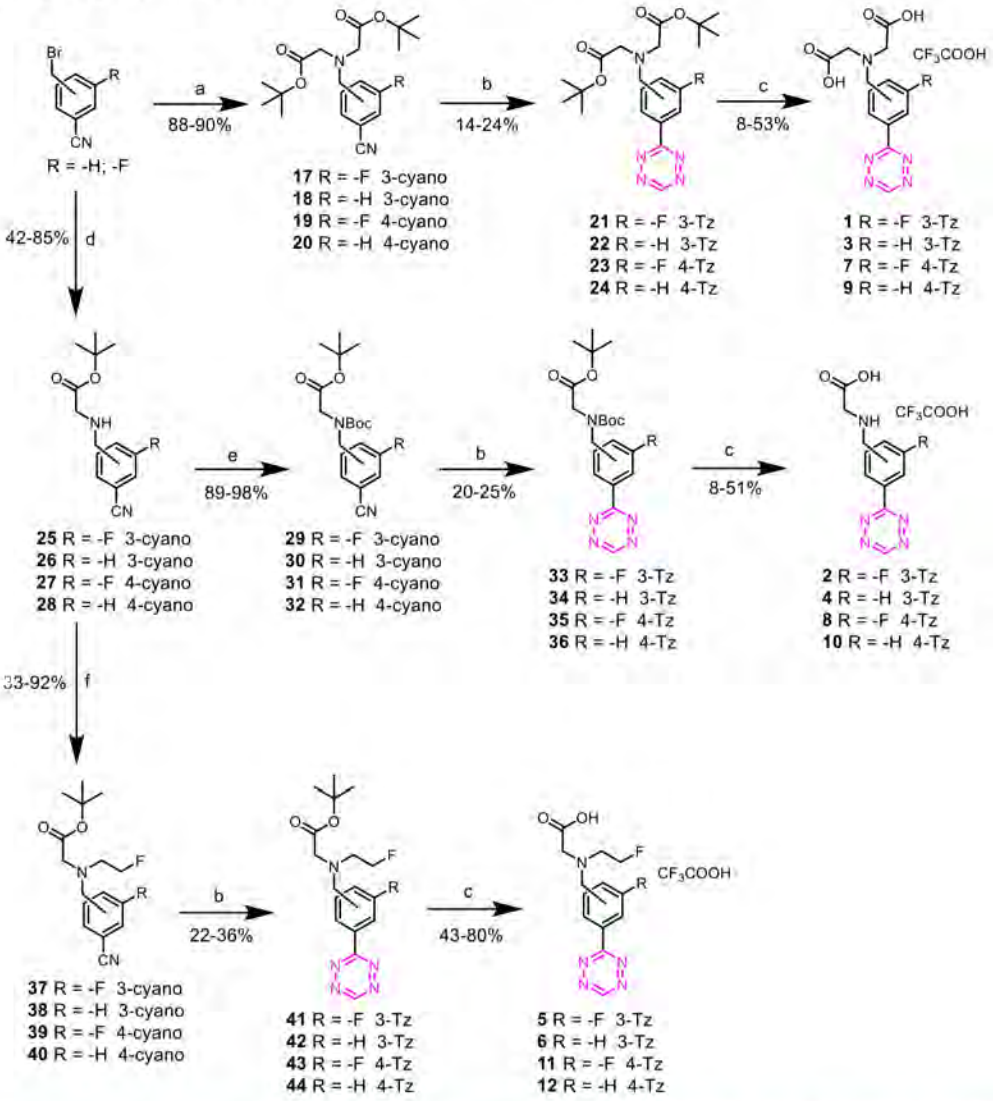
The latter was deprotected under basic conditions and further derivatized to give compounds **50** and **51**. Subsequent Tz formation, deprotection with TFA, and preparative HPLC purification afforded the desired Tzs **13**−**15**.

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

***Ex Vivo* Blocking Assay.** We have recently developed a blocking assay that allows the assessment of the *in vivo* ligation performance of unlabeled Tz derivatives. This assay omits time-consuming development of radiolabeled Tzs for every ligand to be tested. It is based on the ability of Tzs to block the binding of the 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 the literature.[25] The tumor blocking effect of the unlabeled Tz derivatives is determined afterward from the *ex vivo* biodistribution and normalized to the binding of [^{111}In]**16** without any blocking. The setup is displayed in Figure 3.

https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312

Scheme 1. Reagents and Conditions: (a) di-*tert*-Butyl Iminodiacetate, K_2CO_3, CH_3CN, rt, and 12 h; (b) (i) $NH_2NH_2{\cdot}H_2O$, CH_2Cl_2, S_8, EtOH, 50 °C, and 24 h; (ii) $NaNO_2$, AcOH, 0 °C to rt, and 30 min; (c) TFA, CH_2Cl_2, rt, and 2 h; (d) Glycine *tert*-Butyl Ester Hydrochloride, K_2CO_3, CH_3CN, rt, and 12 h; (e) Boc_2O, Et_3N, CH_2Cl_2, rt, and 12 h; and (f) 1-Fluoro-2-iodoethane, K_2CO_3, CH_3CN, Reflux, and 24 h



Investigated Tzs 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 desfluorinated derivatives (**3**, **4**, **6**, **9**, **10**, and **12**) showed a comparable blocking effect to the corresponding 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 the next step, we explored the tolerability of the Tz toward various substituents at position 4 of the phenyl ring. Therefore, we introduced a hydroxy (**13**), methoxy (**14**), and fluoroethoxy (**15**) group at this position. All these compounds showed a blocking effect ≥98%, proving to be among the best Tzs evaluated.

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 five synthesis steps (Scheme 2). Briefly, **45** was converted to the corresponding alcohol and further brominated under radical conditions to yield **56**. The latter was reacted with di-*tert*-butyl iminodiacetate to give **57**. Subsequent Tz formation and nosylation afforded **59**. We decided to use a nosylate leaving group because we have recently reported—for a similar scaffold—that increased radiochemical yields (RCYs) up to 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 (Figure 4A). A protection/deprotection strategy was chosen because unprotected carboxylic acids prevent ^{18}F-fluorinations.[24] Radiolabeling was only possible under low-basicity labeling conditions [[^{18}F]Bu_4NF/Bu_4NOMs PO_4^{3-} and *t*-BuOH/dimethyl sulfoxide (DMSO)].[27] 3-Substituted 1,2,4,5-Tzs are reported to be too sensitive for standard ^{18}F-fluorination approaches.[20] [^{18}F]**15** was labeled in a RCY of 13 ± 2% (n = 3) with a radiochemical purity (RCP) of >98%

https://doi.org/10.1021/acs.jmedchem.1c01326
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Scheme 2. Reagents and Conditions: (a) Ac_2O, Et_3N, CH_2Cl_2, rt, and 12 h; (b) *N*-Bromosuccinimide; AIBN, $CHCl_3$, Reflux, and 12 h; (c) di-*tert*-Butyl Iminodiacetate, Et_3N, CH_3CN, rt, and 12 h; (d) NaOH, H_2O, CH_3CN, rt, and 1 h; (e) (i) $NH_2NH_2 \cdot H_2O$, CH_2Cl_2, S_8, EtOH, 50 °C, and 24 h, and (ii) $NaNO_2$, AcOH, 0 °C to rt, and 30 min; (f) CH_3I or 1-Fluoro-2-iodoethane, K_2CO_3, CH_3CN, Reflux, and 24 h; (g) TFA, CH_2Cl_2, rt, and 2 h; (f) 2-Bromoethanol, NaOH, H_2O, Reflux, and 24 h; and (i) Nosyl Chloride, DIPEA, DMAP, CH_2Cl_2, rt, and 6 h

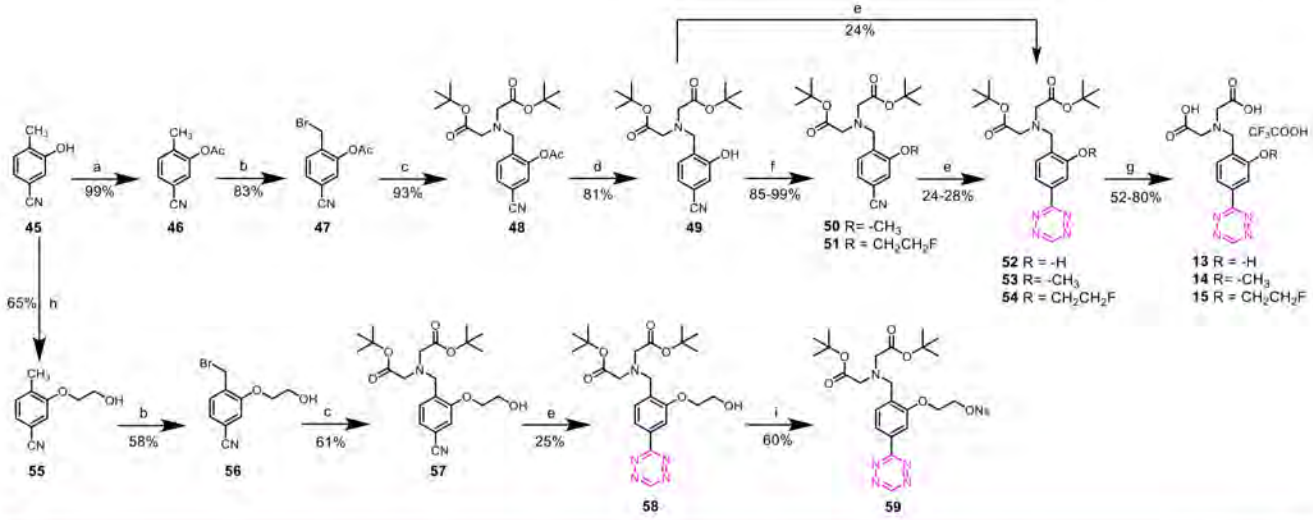

Figure 3. Schematic display of the blocking assay. The blocking effect of non-radiolabeled Tz was determined as the change in tumor uptake of [^{111}In]**16** 22 h p.i. in mice pretreated with CC49-TCO 72 h prior to Tz injection. Each non-radiolabeled Tz was administered 1 h prior to [^{111}In]**16**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

and a molar activity (A_M) of 55 ± 5 GBq/μmol (Figure 4B). The total synthesis time was approximately 90 min including HPLC purification and formulation of the final product. The maximum isolated amount was 1.1 GBq. The reactivity of the Tz core was evaluated by reacting [^{18}F]**15** with *trans*-cyclooctene-*p*-nitrobenzyl ester (TCO-PNB) and monitoring the reaction by radio-HPLC (Figure 4C)

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 h and, therefore, sufficient for most applications (Figure 5A). In contrast, **59** decomposed over time. The process could be prevented by storing **59** at −20 °C as a solid (DMSO solution)—at least for 3 months (Figure 5B).

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 by Rossin and Robillard.[21] Mice were administered with CC49-TCO 72 h prior to administration of [^{18}F]**15**, where 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 was performed. The data are shown in Figure 6 and Table S2. The PET/CT data showed that pretreated mice with CC49-TCO had a significantly higher tumor uptake (1.87 ± 0.31 %IA/g) compared to the control (0.01 ± 0.01 %IA/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) were determined. Uptake in all other tissues was low. The *ex vivo* biodistribution confirmed the results from the imaging experiment (Table S3).

The findings presented here have comparable uptake and ratios to what has been found previously at early timepoints (1−3 h) in pretargeting studies using a similar setup .[26,32,33] There is a tendency that the tumor uptake is somewhat lower when applying a pretargeted imaging, compared to conven-

https://doi.org/10.1021/acs.jmedchem.1c01326
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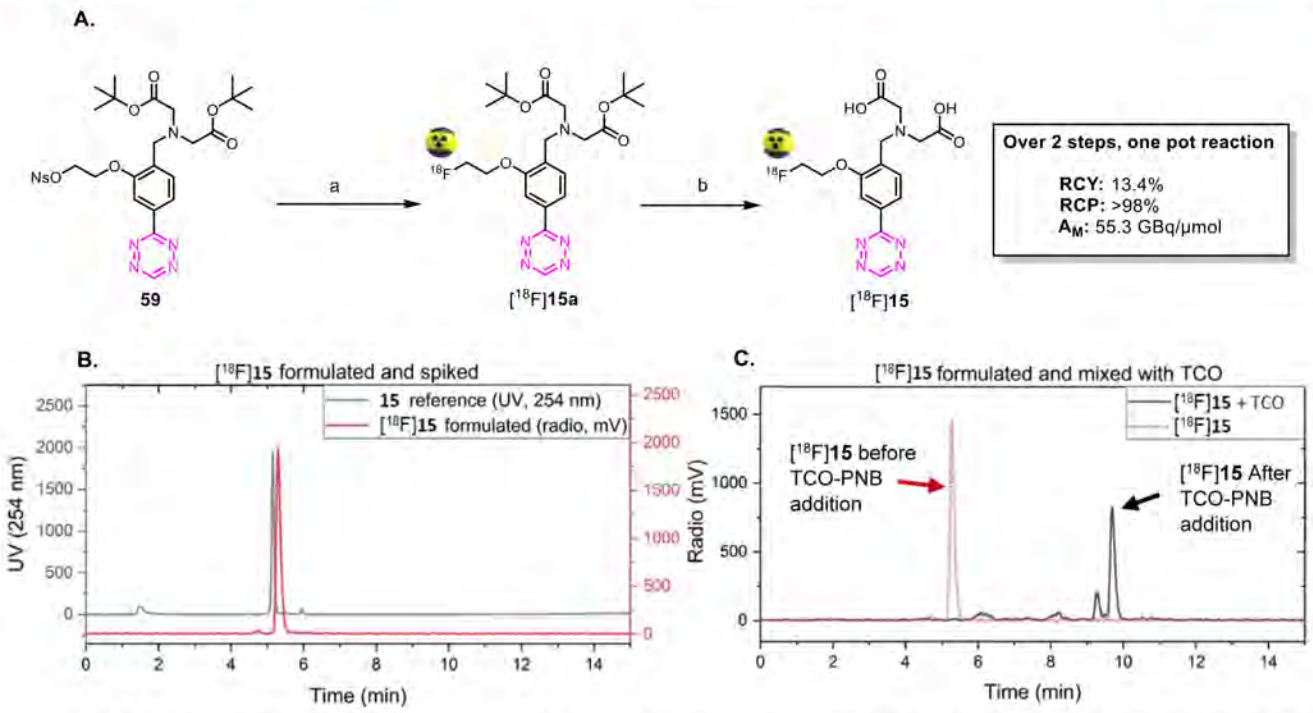

Figure 4. Radiolabeling of [^{18}F]**15**. (A) Reaction sequence. Reagents and conditions: (a) [^{18}F]Bu_4NF/Bu_4NOMs PO_4^{3-}, *t*-BuOH/DMSO, 100 °C, and 5 min and (b) TFA, CH_3CN, 80 °C, and 10 min. (B) Analytical chromatograms of the formulated [^{18}F]**15** spiked with non-radioactive **15** for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

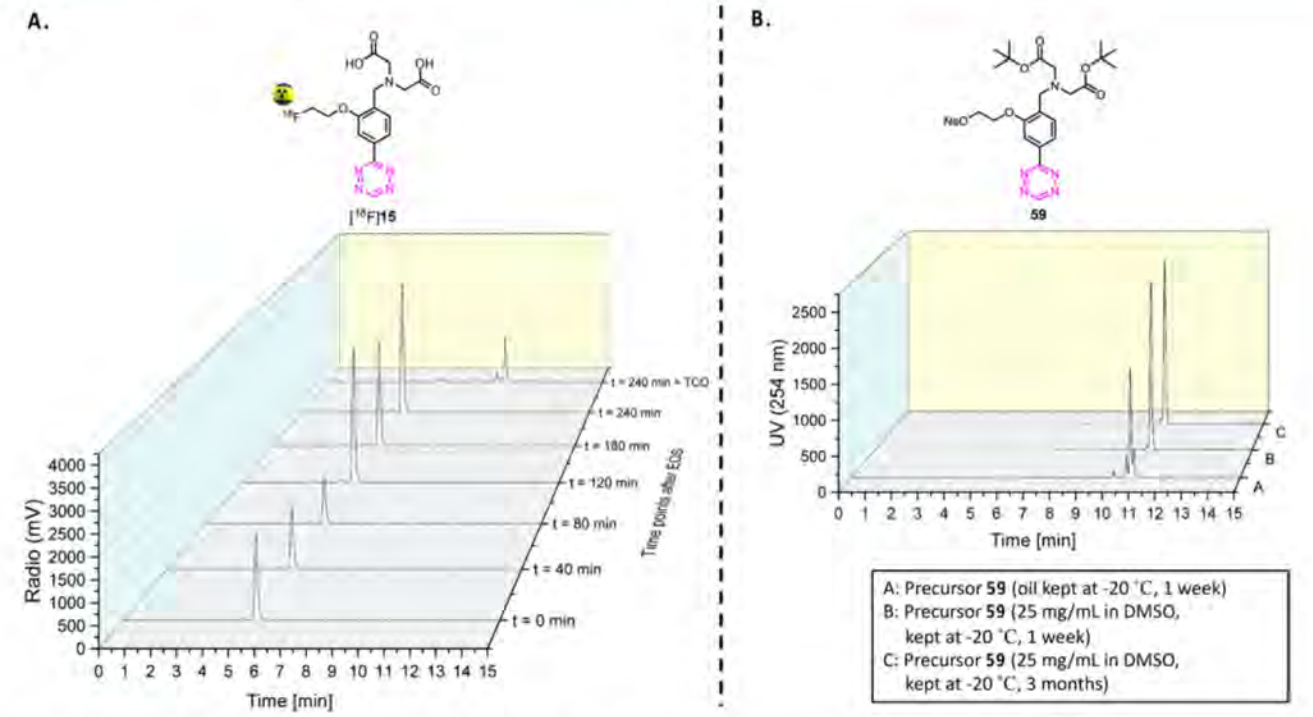

Figure 5. (A) Stability of [^{18}F]**15** at r.t. over 4 h. After 4 h, no signs of decomposition were detected and [^{18}F]**15** was still able to react quantitatively with TCO-PNB. (B) Stability of the precursor **59** as a pure oil at r.t., at −20 °C, or dissolved (25 mg/mL) in DMSO and frozen at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

tional imaging. However, in general, the image contrast is good, and the main obstacle is a relatively low T/B ratio. This phenomenon, a common finding for pretargeting approaches, is likely due to ligation of Tzs with remnant TCO-conjugated antibodies still circulating in the blood.[25,34] However, even though the tumors are clearly visible in studies using

https://doi.org/10.1021/acs.jmedchem.1c01326
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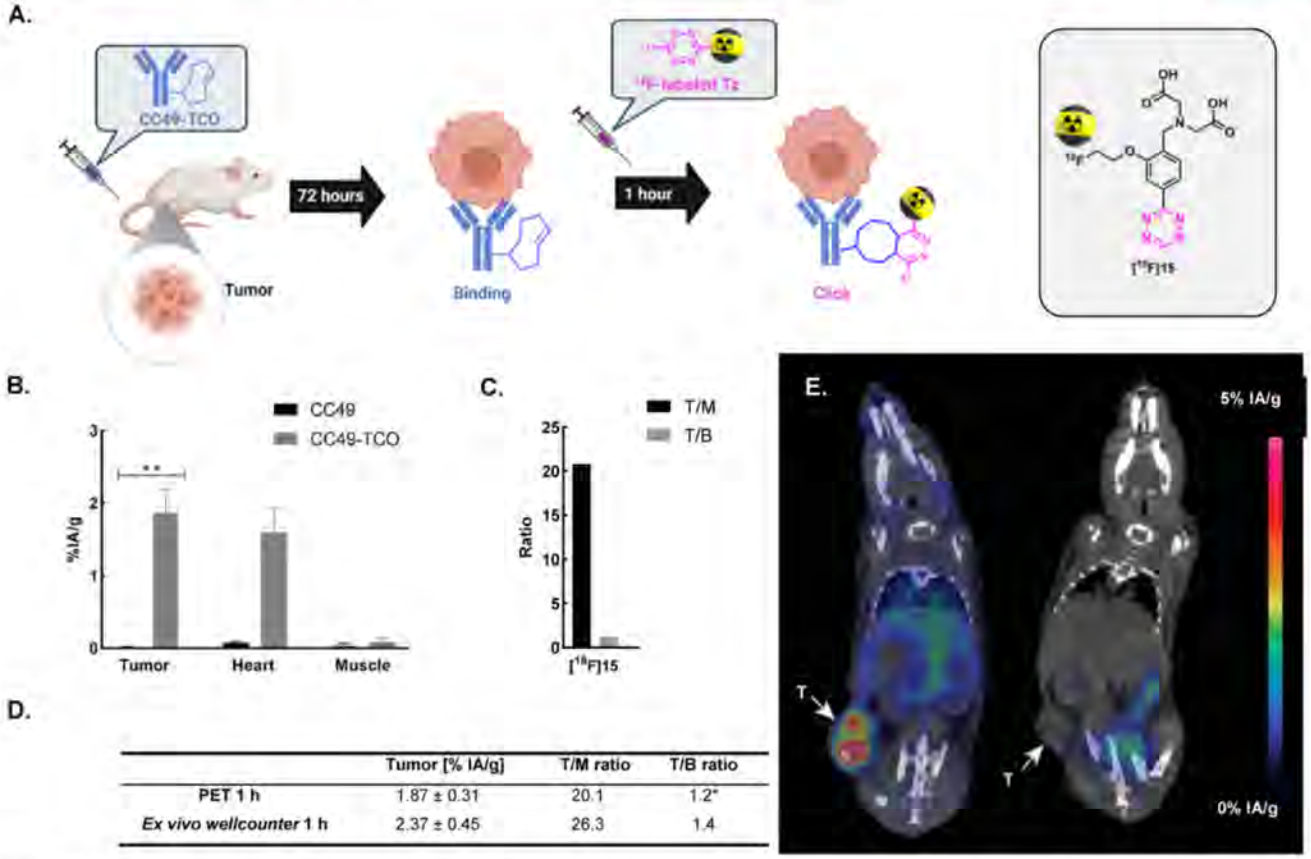

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	Tumor [% IA/g]	T/M ratio	T/B ratio
PET 1 h	1.87 ± 0.31	20.1	1.2*
Ex vivo wellcounter 1 h	2.37 ± 0.45	26.3	1.4

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 %IA/g in tumor, heart, and muscle tissue 1 h p.i. of [^{18}F]**15**. Mean ± S.E.M and n = 4/group. *p < 0.05 (Welch's t-test). (C,D) Image-derived tumor uptake (mean % IA/g), tumor-to-muscle ratio (T/M), and tumor-to-blood ratio (T/B) of [^{18}F]**15**. *Image-derived uptake in the heart from SPECT and PET images used as a surrogate for blood.[26,32,33] (E) Representative images from PET/CT scans 1 h p.i. of [^{18}F]**15**. Mice were administered with either non-modified CC49 (right) or CC49-TCO (left), 72 h prior to [^{18}F]**15** injection. Arrows indicate LS174T tumor xenografts. Scale bar indicates mean %IA/g.

subcutaneous tumors, this might not be the case for tumors located in well-perfused tissue. Therefore, a high blood uptake represents a limitation. The use of clearing agents has previously shown to remove CC49-TCO from blood and increase the T/B ratio drastically.[17,25] If the level of circulating antibodies was decreased, this may also improve tumor uptake as a larger fraction of the administered Tz could potentially reach the tumor. Therefore, going forward, one of the main focus areas should be improving the T/B-ratio, but more knowledge is needed about the use of clearing agents in pretargeting in general. Also, potentially, the circulation time of the antibody could be optimized and the stability of TCO could be improved, thereby allowing for prolonged lag time between administration of the primary and secondary vector; this could also increase the T/B-ratio.

Moreover, pretargeting has at present only been performed successfully *in vivo* using non-internalizing primary vectors, such as the CC49 antibody. This currently is a limitation to the application of the pretargeting concept and needs to be addressed going forward. Future studies will be focused on solving these issues.

CONCLUSIONS

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-Tz 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** has favorable pharmacokinetics and good target-to-background ratios in pretargeting experiments. We believe that [^{18}F]**15** possesses the potential to be clinically translated for *in vivo* pretargeted PET imaging. However, these results are preliminary and need to be confirmed using more complex tumor models (*e.g.*, orthotopic or PDX), which to a higher degree resemble the tumor microenvironment seen in humans. Additionally, studies on improving the T/B ratio are currently ongoing.

EXPERIMENTAL SECTION

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 of K_2CO_3, and 1.25 mL of 10% NaOH in 200 mL of water). Purification of compounds was carried out by column chromatography on silica gel (40−60 μm and 60 Å) or employing CombiFlash NextGen 300+ (Teledyne ISCO). ^{1}H and ^{13}C NMR spectra were recorded on Bruker (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 experiments, the deuterated solvent signal was

https://doi.org/10.1021/acs.jmedchem.1c01326
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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 ^{1}H 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; and 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 a QDa detector. Purification by preparative HPLC was performed on an Agilent 1260 infinity system, column SymmetryPrep-C18, with 17 mL/min H_2O−MeCN and gradient 50−100% for 15 min with 0.1% TFA. 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) 100 Å and 150 × 4.6 mm), eluents: A: H_2O with 0.1% TFA and B: MeCN with 0.1% TFA. Gradient was from 100% A → 100% B over 15 min and back to 100% A over 4 min with a flow rate of 1.5 mL/min. Detection was performed by UV absorption at λ = 254 nm on a UVD 170U detector. Compound **9** and **10** were previously reported by us but are synthesized here 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 and 5.10 mmol) in CH_3CN (30 mL) were added K_2CO_3 (1.06 g and 7.65 mmol) and di-*tert*-butyl iminodiacetate (1.50 g and 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 × 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 and 4.00 mmol), sulfur (0.257 g, 1.00 mmol, and 0.25 equiv), hydrazine monohydrate (1.6 mL and 32.00 mmol), and ethanol (4.0 mL) along with di-*tert*-butyl 2,2′-((3-cyano-5-fluorobenzyl)azanediyl)diacetate (1.55 g and 4.00 mmol) were added to a Biotage microwave vial (10−20 mL) equipped with a stir bar. The vessel was sealed, and the reaction mixture was heated to 50 °C for 24 h, before being allowed to cool to room temperature and unsealed. Then, 3 mL of CH_2Cl_2 and $NaNO_2$ (2.8 g and 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 and 0.36 mmol) in CH_2Cl_2 (5 mL) was added TFA (5 mL). The reaction was stirred at room temperature for 2 h. 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, Tz-H), 8.60 (d, J = 1.4 Hz, 1H, Ar−H), 8.41−8.32 (m, 1H, Ar−H), 7.73−7.64 (m, 1H, Ar−H), 4.59 (s, 2H, $ArCH_2N$), 4.11 (s, 4H, $CH_2N(CH_2)_2$); ^{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: calcd 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 and 15.60 mmol) in CH_3CN (40 mL) were added K_2CO_3 (10.78 g and 78.02 mmol) and glycine *tert*-butyl ester hydrochloride (7.85 g and 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 × 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 and 5.67 mmol) and Et_3N (1.90 mL and 13.62 mmol) in CH_2Cl_2 (40 mL) was added Boc_2O (1.48 g and 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 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 rotamer 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 and 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 rotamer 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 and 0.71 mmol) in CH_2Cl_2 (5 mL)

https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312

was added TFA (5 mL). The reaction was stirred at room temperature for 2 h. 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, Tz-H), 8.52 (s, 1H, Ar−H), 8.30 (ddd, J = 9.4, 2.5, 1.5 Hz, 1H, Ar−H), 7.85−7.25 (m, 1H, Ar−H), 4.36 (s, 2H, $ArCH_2NH$), 3.93 (s, 2H, $ArCH_2NHCH_2$); ^{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: calcd 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 and 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 and 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 and 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, Tz-H), 8.80 (t, J = 1.8 Hz, 1H, Ar−H), 8.70 (dt, J = 7.9, 1.4 Hz, 1H, Ar−H), 7.88 (dt, J = 7.7, 1.4 Hz, 1H, Ar−H), 7.77 (t, J = 7.8 Hz, 1H, Ar−H), 4.71 (s, 2H, $ArCH_2N$), 4.21 (s, 4H, $CH_2N(CH_2)_2$); ^{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: calcd 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 and 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 and 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 rotamer 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 and 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 rotamer 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 and 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, Tz-H), 8.79 (s, 1H, Ar−H), 8.72 (d, J = 7.8 Hz, 1H, Ar−H), 7.85 (d, J = 7.8 Hz, 1H, Ar−H), 7.79 (t, J = 7.7 Hz, 1H, Ar−H), 4.46 (s, 2H, $ArCH_2NH$), 4.03 (s, 2H, $ArCH_2NHCH_2$); ^{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: calcd 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 and 5.23 mmol) and K_2CO_3 (1.83 g and 13.24 mmol) in CH_3CN (40 mL) was added 1-fluoro-2-iodoethane (1.38 g and 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 × 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 and 4.83 mmol) following the procedure employed for 21. 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 and 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,

https://doi.org/10.1021/acs.jmedchem.1c01326
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CD_3OD): δ 10.43 (s, 1H, Tz-H), 8.64 (d, J = 1.6 Hz, 1H, Ar−H), 8.39 (ddd, J = 9.3, 2.5, 1.5 Hz, 1H, Ar−H), 7.69 (dt, J = 9.0, 2.1 Hz, 1H, Ar−H), 5.03−4.94 (m, 1H, CHaF), 4.90−4.83 (m, 1H, CHbF), 4.69 (s, 2H, $ArCH_2N$), 4.15 (s, 2H, $ArCH_2NCH_2$), 3.83−3.71 (m, 1H, $CHaCH_2F$), 3.71−3.60 (m, 1H, $CHbCH_2F$); ^{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: calcd 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 and 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); ^1H 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 and 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); ^1H 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 and 0.86 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.23 g (66%) of **6** as a red solid. ^1H NMR (600 MHz, CD_3OD): δ 10.39 (s, 1H, Tz-H), 8.81 (d, J = 1.8 Hz, 1H, Ar−H), 8.69 (dt, J = 8.0, 1.4 Hz, 1H, Ar−H), 7.89 (dt, J = 7.8, 1.4 Hz, 1H, Ar−H), 7.77 (t, J = 7.8 Hz, 1H, Ar−H), 5.07 (s, 2H, $ArCH_2N$), 5.02−4.96 (m, 1H, CHaF), 4.95−4.85 (m, 1H, CHbF), 4.73 (s, 2H, $ArCH_2NCH_2$), 3.83−3.76 (m, 1H, $CHaCH_2F$), 3.76−3.70 (m, 1H, $CHbCH_2F$); ^{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: calcd 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 and 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); ^1H 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 and 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); ^1H 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 and 0.55 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.02 g (8%) of **7** as a red solid. ^1H NMR (400 MHz, CD_3OD): δ 10.41 (s, 1H, Tz-H), 8.50 (dd, J = 8.0, 1.6 Hz, 1H, Ar−H), 8.38 (dd, J = 10.9, 1.6 Hz, 1H, Ar−H), 7.90 (t, J = 7.7 Hz, 1H, Ar−H), 4.49 (s, 2H, $ArCH_2N$), 3.96 (s, 4H, $CH_2N(CH_2)_2$); ^{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: calcd 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 and 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); ^1H 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 and 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 rotamer mixture). *Rf* = 0.34 (*n*-heptane/EtOAc, 80/20); ^1H 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 and 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 rotamer mixture). *Rf* = 0.36 (*n*-heptane/EtOAc, 80/20); ^1H 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 =

https://doi.org/10.1021/acs.jmedchem.1c01326
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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 and 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. Tz-H), 8.54 (dd, J = 8.1, 1.6 Hz, 1H, Ar−H), 8.45 (dd, J = 10.8, 1.6 Hz, 1H, Ar−H), 7.86 (t, J = 7.7 Hz, 1H, Ar−H), 4.52 (s, 2H, $ArCH_2NH$), 4.07 (s, 2H, $ArCH_2NHCH_2$); ^{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: calcd 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 and 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 and 2.36 mmol) following the procedure employed for **21**. Purification by flash chromatography (85/15 heptane/EtoAc) to yield **24** (0.14 g and 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 and 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, Tz-H), 8.55 (d, J = 8.4 Hz, 2H, Ar−H), 7.68 (d, J = 8.4 Hz, 2H, Ar−H), 4.36 (s, 2H, $ArCH_2N$), 3.89 (s, 4H, $CH_2N(CH_2)_2$). ^{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: calcd 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-bromomethyl-benzonitrile (1.80 g and 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 and 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 rotamer 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 and 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 rotamer 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 and 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, Tz-H), 10.22 (s, 0.4H, Tz-H), 8.52 (d, J = 8.4 Hz, 1.2H, Ar−H), 8.43 (d, J = 8.4 Hz, 0.8H, Ar−H), 7.55 (d, J = 8.1 Hz, 1.2H, Ar−H), 7.33 (d, J = 8.4 Hz, 0.8H, Ar−H), 5.49 (s, 1.2H, $ArCH_2N$), 4.92 (s, 0.8H, $ArCH_2N$), 4.89 (s, 0.8H, $ArCH_2NHCH_2$), 4.13 (s, 1.2H, $ArCH_2NHCH_2$); ^{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: calcd 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 and 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 and 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 rotamer 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 and 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

https://doi.org/10.1021/acs.jmedchem.1c01326
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MHz, CD_3OD): δ 10.37 (s, 1H, Tz-H), 8.39 (ddd, J = 8.1, 2.9, 1.6 Hz, 1H, Ar−H), 8.30−8.24 (m, 1H, Ar−H), 7.67 (t, J = 7.7 Hz, 1H, Ar−H), 4.75 (s, 1H, ArCHaN), 4.73 (s, 1H, ArCHbN), 4.68−4.60 (m, 1H, CHaF), 4.60−4.52 (m, 1H, CHbF), 4.45−4.39 (m, 1H, $CHaCH_2F$), 4.39−4.31 (m, 1H, $CHbCH_2F$), 4.13 (s, 1H, $ArCH_2NCHa$), 4.12 (s, 1H, $ArCH_2NCHb$); ^{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: calcd 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 and 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); ^1H 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 and 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); ^1H 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 and 0.43 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.14 g (80%) of **12** as a red solid. ^1H NMR (400 MHz, CD_3OD): δ 10.40 (s, 1H, Tz-H), 8.69 (d, J = 8.4 Hz, 2H, Ar−H), 7.85 (d, J = 8.4 Hz, 2H, Ar−H), 5.04−4.97 (m, 1H. CHaF), 4.92−4.86 (m, 1H, CHbF), 4.70 (s, 2H, $ArCH_2N$), 4.19 (s, 2H, $ArCH_2NCH_2$), 3.83−3.75 (m, 1H, $CHaCH_2F$), 3.75−3.66 (m, 1H, $CHbCH_2F$); ^{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: calcd 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 and 23.28 mmol) and Et_3N (9.74 mL and 7.07 mmol) in CH_2Cl_2 (30 mL) was added acetic anhydride (2.64 mL and 27.93 mmol). The resulting mixture was stirred at room temperature for 12 h. The organic layer was then washed with water (2 × 30 mL) and brine (2 × 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); ^1H 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 and 17.12 mmol) and *N*-bromosuccinimide (4.57 g and 25.68 mmol) in $CHCl_3$ (50 mL) was added AIBN (1.12 g and 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 × 30 mL) and brine (2 × 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); ^1H 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 and 14.17 mmol) in CH_3CN (50 mL) were added Et_3N (5.92 mL and 42.51 mmol) and di-*tert*-butyl iminodiacetate (3.65 g and 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 × 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); ^1H 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 and 13.14 mmol) in CH_3CN (50 mL) was added a 1 M 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 1 M HCl solution. The resulting slurry was extracted with EtOAc (3 × 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); ^1H 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 and 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); ^1H 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 and 0.21 mmol) following the procedure employed for **1**. Purification by preparative HPLC afforded 0.065 g (72%) of **13** as a red solid. ^1H NMR (400 MHz, DMSO): δ 10.57 (s, 1H, Tz-H), 8.89−7.81 (m, 2H, Ar−H), 7.48 (d, J = 7.9 Hz, 1H, Ar−H), 4.03 (s, 2H, $ArCH_2N$), 3.56 (s, 4H, $ArCH_2N(CH_2)_2$); ^{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: calcd 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 and 1.07 mmol) and K_2CO_3 (0.44 g and 3.19 mmol) in CH_3CN (10 mL) was added CH_3I (0.07 mL and 1.17 mmol). The reaction was refluxed for 12 h and then concentrated under reduced pressure. The resulting mixture was

https://doi.org/10.1021/acs.jmedchem.1c01326
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diluted with water (20 mL), extracted with EtOAc (3 × 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 and 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 and 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, Tz-H), 8.39−8.11 (m, 2H, Ar−H), 7.75 (d, *J* = 7.9 Hz, 1H, Ar−H), 4.77 (s, 2H, $ArCH_2N$), 4.24 (s, 4H, $ArCH_2N(CH_2)_2$), 4.09 (s, 3H, OCH_3); ^{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*: calcd 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 and 6.97 mmol) and K_2CO_3 (1.92 g and 13.94 mmol) in CH_3CN (50 mL) was added 1-fluoro-2-iodoethane (1.33 g and 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 × 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 and 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 and 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, Tz-H), 8.31−8.22 (m, 2H, Ar−H), 7.76 (d, *J* = 7.8 Hz, 1H, Ar−H), 5.02−4.93 (m, 1H, CHaF), 4.92−4.82 (m, 1H, CHbF), 4.74 (s, 2H, $ArCH_2N$), 4.60−4.56 (m, 1H, $CHaCH_2F$), 4.54−4.42 (m, 1H, $CHbCH_2F$), 4.21 (s, 4H, $ArCH_2N(CH_2)_2$); ^{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*: calcd 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 and 10.0 mmol) was dissolved in NaOH aqueous solution (25 mL and 15.0 mmol), and 2-bromoethanol (1.06 mL and 15.0 mmol) was added. The resulting mixture was heated at 90 °C for 12 h. The reaction was then cooled to room temperature, diluted with water (30 mL), and extracted with CH_2Cl_2 (2 × 50 mL). The organic layer was washed with 10% NaOH (2 × 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 and 7.33 mmol) and *N*-bromosuccinimide (1.43 g and 8.07 mmol) in $CHCl_3$ (40 mL) was added AIBN (0.48 g and 2.93 mmol). The reaction was refluxed for 24 h. The solvent was removed under a vacuum, and the crude was 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 and 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 and 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 and 0.19 mmol) and DIPEA (0.13 mL and 0.75 mmol) in CH_2Cl_2 (20 mL) were added nosyl chloride (0.12 g and 0.57 mmol) and DMAP (0.005 g and 0.04 mmol). The reaction was stirred at room temperature for 4 h. The

https://doi.org/10.1021/acs.jmedchem.1c01326
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solvent was then evaporated under reduced pressure. Purification by flash chromatography (*n*-heptane/EtOAc,75/25) 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, Tz-H), 8.41 (d, J = 8.8 Hz, 2H, Ar−H Nos), 8.27 (dd, J = 8.0, 1.5 Hz, 1H, Ar−H), 8.18 (d, J = 8.8 Hz, 2H, Ar−H Nos), 7.96 (d, J = 1.5 Hz, 1H, Ar−H), 7.80 (d, J = 8.0 Hz, 1H, Ar−H), 4.56 (dd, J = 5.7, 3.4 Hz, 2H, CH_2ONos), 4.36 (dd, J = 5.7, 3.4 Hz, 2H, CH_2CH_2ONos), 3.97 (s, 2H, $ArCH_2N$), 3.47 (s, 4H, $ArCH_2N(CH_2)_2$), 1.46 (s, 18H, 2 x $C(CH_3)_3$); ^{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: calcd for $[C_{29}H_{37}N_6O_{10}S]^+$, 661.23; found, 661.24.

Reaction Kinetics Measurement. Stopped-flow measurements were performed using an SX20-LED stopped-flow spectrophotometer (Applied Photophysics) equipped with a 535 nm LED (optical pathlength 10 mm and full width half-maximum 34 nm) to monitor the characteristic Tz visible light absorbance (520−540 nm). The reagent syringes were loaded with a solution of axTCO-PEG$_4$, and the instrument was primed. The subsequent data were collected in triplicate to sextuplicate for each Tz. Reactions were conducted at 37 °C in DPBS and recorded automatically at the time of acquisition. The data sets were analyzed by fitting an exponential decay using Prism 6 (GraphPad) to calculate the observed pseudo-first-order rate constants that were converted to second-order rate constants by dividing with the concentration of the excess TCO compound. Observed 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 week old female nude BALB/c mice (Charles River, Sulzfeld, Germany) were allowed to acclimatize for 1 week. At all time, the animals had access to water and chow ad libitum. The 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 of 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 = 1/2 (length × width^2).

Blocking Experiments. Tumor bearing animals were matched in groups based on their tumor volume (tumor volumes of ∼100−300 mm^3 and 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 and 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 the tumor, blood, heart, lung, liver, spleen, kidney, and muscle tissue were resected and weighted, and the radioactivity was measured using a gamma counter (Wizard2, PerkinElmer). The data were 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 using 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 system equipped with a C18 column (Luna 5 μm C18(2) 100 Å and 150 mm × 4.6 mm). Eluents: A, H_2O with 0.1% TFA and B, MeCN with 0.1% TFA. Gradient was from 100% A to 100% B over 15 min and back to 100% A over 4 min with a flow rate of 1.5 mL/min. Detection was performed 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 of 0.5 M K_3PO_4 and washing it with 10 mL of H_2O afterward. [^{18}F]F$^-$ was eluted from the QMA into a 4 mL v-shaped vial with 1 mL of 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 of DMSO and then diluted with 833 μL of tBuOH. The solution was added to the dried fluoride solution and allowed to react for 5 min at 100 °C. The reaction was cooled to 50 °C in air before addition of 3 mL of 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 of EtOH followed by 10 mL of H_2O. The SPE cartridge was flushed with another 5 mL of H_2O and dried with N_2. The product was eluted from the SPE cartridge with 2 mL of MeCN in a 7 mL v-shaped vial containing 600 μL of 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 was 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.

Tz Core Reactivity test. The reaction between [^{18}F]**15** and TCO-PNB was performed by mixing the formulated [^{18}F]**15** (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 analyzed an analytical HPLC system.

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 of TCO-modified CC49 (CC49-TCO, ∼7 TCO/mAb, 2 nmol TCO/mouse), or non-modified CC49 (control) (n = 4 per group). 72 h later, [^{18}F]**15** [1.74 ± 0.319 (mean ± SD) MBq/100 μL (55.3 GBq/μmol)] was injected i.v., followed by a PET/CT scan (Inveon, Siemens Medical Solutions), 1 hour post injection. The PET data were 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 were created on the full CT tumor volume, as well as the heart and muscle tissue, to quantify uptake of [^{18}F]**15** (Figure 6 and Table S2). Additionally, selected organs were harvested for the *ex vivo* biodistribution. The radioactivity in the tissues were determined using a gamma counter (Wizard2, PerkinElmer). The data were 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 %IA/g values was analyzed using Welch's T-test. Results were considered significant when $p < 0.05$.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c01326.

Radiochemistry and PET data, NMR spectra, and HPLC-UV chromatograms (PDF)

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https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312

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Author Contributions

[#]U.M.B. and K.B. contributed equally to the work. Organic synthesis was performed by U.M.B. and subsequent radiolabeling experiments were performed by K.B. *In vivo* studies and PET experiments were performed by J.J., L.H., and V.S. H.M. evaluated the reaction kinetics of all the compounds in the article. The study was conceptionally designed by all the authors and the article was written by U.M.B., K.B., and M.M.H. with contribution from all authors. All authors have given approval to the final version of the article.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

V.S. was supported by the BRIDGE—Translational Excellence Programme at the Faculty of Health and Medical Sciences, University of Copenhagen, funded by the Novo Nordisk Foundation (grant agreement no. NNF18SA0034956). The Lundbeck Foundation, the Novo Nordisk Foundation, the Innovation Fund Denmark, the Carlsberg Foundation (CF18-0126), and the Research Council for Independent Research (grant agreement no. 8022-00187B) are further acknowledged. The modified antibody used in this study was kindly provided by Tagworks Pharmaceuticals.

■ ABBREVIATIONS

A_M, molar activity; DPBS, Dulbecco's phosphate buffered saline; ^{18}F, fluorine-18; ^{111}In, indium-111; PDX, patient derived xenograft; PET, positron-emission-tomography; PET/CT, positron emission tomography computed tomography; RCP, radiochemical purity; RCY, radiochemical yield; T/B, tumor-to-blood; TCO, *trans*-cyclooctene; TCO-PNB, *trans*-cyclooctene-*p*-nitrobenzyl ester; T/M, tumor-to-muscle; Tz, tetrazine

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https://doi.org/10.1021/acs.jmedchem.1c01326
J. Med. Chem. 2021, 64, 15297−15312