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Preparation and evaluation of 99mTc-labeled tridentate chelates for pre-targeting using bioorthogonal chemistry

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Abstract:	Pre-targeting combined with bioorthogonal chemistry is emerging as an effective way to create new radiopharmaceuticals. Of the methods available, the inverse electron demand Diels-Alder (IEDDA) cycloaddition between a radiolabeled tetrazine and trans-cyclooctene (TCO) linked to a biomolecule has proven to be a highly effective bioorthogonal approach to imaging specific biological targets. Despite the fact that technetium-99m remains the most widely used isotope in diagnostic nuclear medicine, there is a scarcity of methods for preparing 99mTc-labeled tetrazines. Herein we report the preparation of a family of tridentate-chelate-tetrazine derivatives and their Tc(I) complexes. These hitherto unknown compounds were radiolabeled with 99mTc using a microwave-assisted method in 31 to 83 % radiochemical yield. The products are stable in saline and PBS and react rapidly with TCO derivatives in vitro. Their in vivo pre-targeting abilities were demonstrated using a TCO-bisphosphonate (TCO-BP) derivative that localizes to regions of active bone metabolism or injury. In murine studies, the 99mTc-tetrazines showed high activity concentrations in knees and shoulder joints, which was not observed when experiments were performed in the absence of TCO-BP. The overall uptake in non-target organs and pharmacokinetics varied greatly depending on the nature of the linker and polarity of the chelate.
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TITLE:
Preparation and evaluation of ^{99m}*Tc-labeled tridentate chelates for pre-targeting using bioorthogonal chemistry*

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KEYWORDS:
^{99m}Tc, tetrazine, *trans*-cyclooctene, bioorthogonal chemistry, pre-targeting, imaging.

SHORT ABSTRACT:
Here, we describe a protocol for radiolabeling and *in vivo* testing of tridentate ^{99m}Tc(I) chelate-tetrazine derivatives for pre-targeting and bioorthogonal chemistry.

LONG ABSTRACT:
Pre-targeting combined with bioorthogonal chemistry is emerging as an effective way to create new radiopharmaceuticals. Of the methods available, the inverse electron demand Diels-Alder (IEDDA) cycloaddition between a radiolabeled tetrazines and *trans*-cyclooctene (TCO) linked to a biomolecule has proven to be a highly effective bioorthogonal approach to imaging specific biological targets. Despite the fact that technetium-99m remains the most widely used isotope in diagnostic nuclear medicine, there is a scarcity of methods for preparing ^{99m}Tc-labeled tetrazines. Herein we report the preparation of a family of tridentate-chelate-tetrazine derivatives and their Tc(I) complexes. These hitherto unknown compounds were radiolabeled with ^{99m}Tc using a microwave-assisted method in 31% to 83% radiochemical yield. The products are stable in saline and PBS and react rapidly with TCO derivatives *in vitro*. Their *in vivo* pre-targeting abilities were demonstrated using a TCO-bisphosphonate (TCO-BP) derivative that localizes to regions of active bone metabolism or injury. In murine studies, the ^{99m}Tc-tetrazines showed high activity concentrations in knees and shoulder joints, which was not observed when experiments were performed in the absence of TCO-BP. The overall uptake in non-target organs and pharmacokinetics varied greatly depending on the nature of the linker and polarity of the chelate.

INTRODUCTION:
^{99m}Tc remains the dominant radioisotope used in diagnostic nuclear medicine, with over 50 million imaging procedures conducted per year worldwide[1–3]. The majority of ^{99m}Tc agents used clinically are perfusion type radiopharmaceuticals. There are a limited number of actively targeted compounds in which ^{99m}Tc is directed to bind a specific biomarker through ligation to a targeting construct. The creation of targeted ^{99m}Tc radiopharmaceuticals is often hindered by the influence of ^{99m}Tc-ligand complexes on the ability of the targeting molecule to bind the biomarker of interest, or the isotopes half-life is not long enough for use with higher molecular weight biomolecules such as antibodies. The latter typically requires several days before images are acquired in order for the biomolecule to clear from non-target tissues. Pre-targeting offers an alternative approach to overcome these challenges.

Pre-targeting combined with bioorthogonal chemistry has been shown to be an effective way to develop new molecular imaging probes for both fluorescence and radio-imaging[4–8]. The inverse electron demand Diels-alder (IEDDA) reaction between 1,2,4,5-tetrazine (Tz) and *trans*-cyclooctene (TCO) derivatives, as shown in Figure 1, has been shown to be particularly effective[6]. The IEDDA reaction with these components can exhibit fast kinetics in PBS ($k_2 \approx 6000$ M^{-1} s^{-1}) and high selectivity, making it ideal for *in vivo* pre-targeting applications[9,10].

The most common approach used involves administering a TCO-derived targeting vector and following a sufficient delay period, a radiolabeled tetrazine is administered. Radiolabeled

tetrazines based on ^{11}C, ^{18}F, ^{64}Cu, ^{89}Zr, and ^{111}In have been reported[11–15]. In contrast, there is only one report of a ^{99m}Tc-labeled Tz, which was prepared using a HYNIC type ligand requiring the use of co-ligands to prevent protein binding and degradation *in vivo*[16]. As an alternative, we report here the synthesis of $^{99m}Tc(I)$ labeled tetrazines using a family of ligands which form stable tridentate complexes with a $[^{99m}Tc(CO)_3]^+$ core.

[please place Figure 1 here]

The family of ligands prepared contain tridentate chelates that vary in polarity and the nature of the linker group between the metal binding region and the Tz (Figure 2). The goal was to identify a ^{99m}Tc-Tetrazine construct that could effectively localize and react with TCO-labeled sites *in vivo* and rapidly clear when not bound, in order to yield high target-to-non-target ratios. To test the ligands, a TCO-derivative of a bisphosphonate (TCO-BP) was used[17]. We have shown previously that TCO-BP localizes to areas of active bone metabolism and can react with radiolabeled tetrazines *in vivo*[18]. It is a convenient reagent to test new tetrazines, because it can be prepared in a single step and experiments can be performed in normal mice where localization occurs primarily in the joints (knees and shoulders).

PROTOCOL:

Animal studies were approved by the Animal Research Ethics Board at McMaster University in accordance with Canadian Council on Animal Care (CCAC) guidelines.

1. Radiolabeling of Tz-tridentate ligands with ^{99m}Tc.

CAUTION: The following procedures require the use of radioactive compounds. Work should only be done in a licensed laboratory with adherence to safety and disposal regulations. Microwave reactions should be performed in a microwave specifically designed for chemical synthesis.

1.1) Synthesis of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ [19,20]

1.1.1) In a microwave vial, combine 8 mg $K_2[BH_3CO_2]$, 15 mg Na_2CO_3, 20 mg $Na_2B_4O_7{\cdot}10H_2O$, and 25 mg $KOCO[CH(OH)]_2COONa{\cdot}4H_2O$. Purge the vial for 10 min with argon gas.

1.1.2) Add 4 mL of $^{99m}TcO_4^-$ (~ 1100 MBq, ~30 mCi) in 0.9% saline to the vial.

1.1.3) Heat the reaction in a microwave for 3.5 min at 110 °C after 10 s of stirring to ensure thorough mixing of reagents.

1.1.4) Adjust the pH of the solution to 3.5-4 using ~400 µL of 1 M HCl. Verify using pH paper.

1.2) Radiolabeling of Tetrazine ligands 1-5

1.2.1) Dissolve 2 mg of each ligand (compounds **1-5**) in 250 µL MeOH[21].

1.2.2) Add 250 µL of $[^{99m}Tc(CO)_3(H_2O)_3]^+$ (~ 74 MBq, ~ 2 mCi) to each solution.

1.2.3) Heat the reaction mixture using a microwave for 20 min at 60 °C.

NOTE: This step was identical for all 5 tetrazines.

1.2.4) For compounds **2**-**5**, evaporate the solvent and re-dissolve the resulting products in 1 mL of 1:1 v/v DCM:TFA.

1.2.5) Heat the dissolved reaction products (**2-5**) at 60 °C in a microwave for 6 min (**2-4**) or 10 min (**5**).

1.2.6) After cooling to room temperature, evaporate the solvent using an evaporator (36 °C, 8 mbar, 3 min, 6000 rpm) and dissolve the dried compound in 1:1 ACN:H_2O or 1:1 MeOH:H_2O, prior to HPLC purification.

1.2.7) Purify the ^{99m}Tc-labeled compounds (**1-5**), including separating the labeled product from unlabeled tetrazine ligand, using HPLC (C_{18} reversed-phase). Typically, use an elution gradient of 30:70 ACN:H_2O (both with 0.1% TFA) to 40:60 ACN:H_2O over 20 min (18 min) and a C_{18} analytical 4.6 x 100 mm column. Use both UV (254 nm) and gamma detection.

1.2.7.1) Take a small sample of each labeled product and compare its HPLC retention time to that of a co-injected, non-radioactive, Re-labeled standard (0.125 mg in 20% methanol-H_2O). The Re-labeled standard is identified in the UV HPLC trace, and will elute at the same time as the ^{99m}Tc-labeled compound in the γ-HPLC trace. This co-injection shows peaks at comparable retention times, confirming the identity of the ^{99m}Tc-labeled compound.

1.2.8) Evaporate the solvent from HPLC fractions using an evaporator (36 °C, 8 mbar, 3 min, 6000 rpm).

1.2.9) Formulate the purified compound at a concentration of 7.4 kBq/μL in PBS, containing 0.5% BSA and 0.01% Tween-80.

1.2.10) To ensure the labelled compounds are stable, perform an *in vitro* stability study. Incubate the formulated compound at 37 °C for 1, 4 and 6 hours, injecting a small amount (3.7 MBq) of the mixture on the HPLC at each time point to assess stability.

2. Pre-targeted Bio-distribution Studies

2.1) Preparation of animals

2.1.1) Using 7-9 week old, female Balb/c mice (n=3), administer TCO-BP formulated in saline (20 mg/kg) (5 μg/μL), via tail-vein injection.

2.1.2) Place mouse in physical restraint device, and identify the veins located on the lateral surfaces of the tail and wipe with an alcohol swab. At approximately 2 cm from the end of the

tail, insert a 30-gauge needle at a shallow angle, parallel to the vein. Slowly depress the plunger to inject, remove needle and apply clean gauze sponge at injection site with slight pressure until bleeding stops.

2.1.3) At 1 h post injection of TCO-BP, administer ~0.74 MBq (20 μCi) of ^{99m}Tc-tetrazine formulated in 100 μL of 0.5% BSA, 0.01% Tween-80 in PBS, via tail-vein injection.

2.2) Bio-distribution studies

2.2.1) At the desired time point (t = 6 h), anaesthetize the mice using 3% isoflurane and 2% oxygen gas mixture.

2.2.2) Collect blood (1 mL) via cardiac puncture using a syringe pre-treated with heparin. Place mouse on its back with nose in the nose cone for continued anesthesia and locate the xiphoid process on the animal.

2.2.2.1) Insert a 25-gauge needle, slightly to the left of the animal's midline under the xiphoid process, at a 20-degree angle. Fully insert the needle, and slowly pull back on the plunger to see blood in the needle hub if the heart was punctured. Slightly readjust the needle while holding the plunger if necessary, to puncture the heart. Slowly draw blood into the syringe.

2.2.3) Euthanize the animal by cervical dislocation, while under anesthesia.

2.2.4) Place each animal in a plastic bag and use a dose calibrator (^{99m}Tc setting) to measure the whole body activity level.

2.2.5) Collect the following tissues and fluids in pre-weighed counting tubes: blood, bone (knee and shoulder), gall bladder, kidneys, liver, stomach (with contents), small intestines (with contents), large intestines and caecum (with contents), thyroid and trachea, urinary bladder with urine, and tail.

2.2.6) Rinse appropriate tissues (excluding blood, gall bladder, and urinary bladder) in PBS to remove blood and blot dry before placing the tissues in appropriate counting tubes.

2.2.7) Place animal carcass in a plastic bag and measure residual whole body activity using a dose calibrator.

2.2.8) Weigh each tube containing a tissue sample. Subtract initial weight of the tube to obtain mass of the tissue.

2.2.9) Use a dose calibrator (^{99m}Tc setting) to measure the amount of activity in a test sample (100 μL) at the time of injection for each mouse.

NOTE: This test sample is equal to the injection volume, thus giving the activity count at the time of injection.

2.2.10) At the time of tissue measurement, aliquot 5 µL of the test sample used previously. Use a multi-detector gamma counter (^{99m}Tc setting) and count to obtain the count per minute (CPM) for the 5 µL test sample.

2.2.11) Use the two values obtained in 2.2.9 and 2.2.10 to calculate the activity and CPM relationship using equation *1* to obtain a conversion factor (CPM µCi^{-1}).

$$\frac{\boldsymbol{Standard\ CPM} * \left(\frac{\boldsymbol{dose\ cal\ volume}}{\boldsymbol{gamma\ counter\ volume}}\right)}{\boldsymbol{\mu Ci\ in\ Standard\ at\ time\ of\ injection}} \quad \textit{(1)}$$

2.2.12) Use the gamma counter to measure the amount of radioactivity in each tissue or fluid sample.

2.2.13) Use equation 1 to calculate the amount of activity in each tissue or fluid at the time of measurement relative to the total injected dose. This value is then normalized by organ weight and reported as percent injected dose per gram (i.e. %ID/g) of tissue.

2.2.14) Follow steps 2.1.2 to 2.2.13 to conduct a negative control experiment using the ^{99m}Tc-labeled tetrazine ligands in the absence of TCO-BP. Sacrifice mice (n=3) at 0.5, 1, 4 and 6 h post injection and obtain tissue or fluid as described above.

REPRESENTATIVE RESULTS:

The ligands were synthesized using different linkers and chelators via a simple reductive amination strategy (Figure 2), followed by coupling of the product to a commercially available tetrazine[22,23]. Radiolabeling was performed using the same method for all compounds and was highly reproducible. The process was optimized by varying the pH, amount of ligand, reaction time and temperature whereupon the ^{99m}Tc-radiolabeled compounds **1-5** were obtained in moderate to high radiochemical yield: 83% (**1**), 45% (**2**), 31% (**3**), 42% (**4**), and 54% (**5**). Following HPLC purification from unreacted ligand and evaporation using an evaporator, the compounds were formulated in PBS containing 0.5% BSA and 0.01% Tween80 prior to injection. The specific activity of the purified ^{99m}Tc-labeled tetrazine was ~1.48 MBq/µg. Studies were conducted to assess the stability of the ^{99m}Tc-labeled tetrazine ligands prior to *in vivo* studies. The stability was monitored by HPLC at 1, 4 and 6 h with no visible degradation over 6 h (R_t= 14 min), as seen in Figure 3 for compound **4** as an example.

[please place Figure 2 here]

[please place Figure 3 here]

For the *in vivo* testing, healthy Balb/c mice were used. Briefly, for each compound, groups of

mice (n=3) were injected with TCO-BP (100 μL, 20 mg/kg), which was followed by administration of the ^{99m}Tc-labeled compounds 1 h later. At 6 h post-injection of the ^{99m}Tc complexes, the animals were sacrificed and the activity concentrations in various tissues and fluids determined. The resulting data is reported as percent injected dose per gram tissue (%ID/g) and is shown in Figure 4. Representative ratios of bone (knee or shoulder) to blood for each of the five ^{99m}Tc-labeled Tz compounds are shown on Table 1. These data indicate clearly that compound **3** provided optimal targeting combined with clearance from blood, and that there was substantial variation among the ^{99m}Tc-labeled compounds in regard to off-target tissue localization. A negative control study using CD1 mice (n=3) was conducted, where mice were injected with ^{99m}Tc-tetrazine ligands in the absence of TCO-BP. Mice were sacrificed at 0.5, 1, 4 and 6 h and %ID/g was determined for all tissues and fluids. For all compounds tested, where data for compound **2** is presented in Figure 5, no significant uptake was seen in bone or other tissues (heart, lungs, spleen, skeletal muscle) not shown in Figure 4.

[please place Figure 4 here]

[please place Figure 5 here]

Figure 1. The bioorthogonal IEDDA reaction between tetrazine and *trans*-cyclooctene.

Figure 2. Compounds **1-5** were produced using different linkers (Y) and chelators (X) as shown (bottom). All compounds were radiolabeled with $[^{99m}Tc(CO)_3(H_2O)_3]^+$ using the same reaction conditions (top), with the exception of **1**, which did not require step (ii).

Figure 3. Stability test results using compound **4**. γ-HPLC traces of **4** incubated in PBS at 37 °C for 1, 4 and 6 h.

Figure 4. Bio-distribution results for ^{99m}Tc-labeled tetrazine derivatives **1-5** (bars indicated). Data shown were obtained from selected tissues and fluids taken 6 h post injection of the radiolabeled derivatives, and activity was normalized to tissue or fluid weight, as mean percent injected dose per gram of tissue or fluid (%ID/g) ± SEM. Bone targets are indicated by ^. NOTE: All remaining tissues not shown had mean%ID/g that was less than 1%.

Figure 5. Bio-distribution results for control study using ^{99m}Tc-labeled tetrazine (**2**) without prior injection of TCO-BP. Data shown were obtained from selected tissues and fluids taken from 3 mice at 0.5, 1, 4, and 6 h post injection of **2**. Activity was normalized to tissue or fluid weight, as mean percent injected dose per gram of tissue or fluid (%ID/g) ± SEM.

Table 1. Bone tissue: blood ratios determined from bio-distribution studies.

DISCUSSION:

A collection of tetrazine-linked tridentate chelates of varying polarities was prepared, and the utility of their ^{99m}Tc complexes in the IEDDA reaction with a TCO derivative *in vivo* was assessed. An effective and reproducible ^{99m}Tc labeling method was developed for five tetrazine-chelates,

where the ligand concentration was 10^{-3} M. The labeling step was followed by deprotection of *t*-butyl groups (for compounds **2-5**). The high concentration of ligand was used to improve the radiochemical yield and reduce reaction times which minimized degradation of the tetrazine[21]. The product was isolated and separated from unlabeled ligand and any radiochemical impurities by HPLC, resulting in radiochemical yields ranging from 31-83%, with all having >99% radiochemical purity and a high specific activity of ~1.48 MBq/µg. All compounds were shown to be stable in PBS containing 0.5% BSA and 0.01% Tween80 for up to 6 h (Figure 3).

Bisphosphonate compounds, like TCO-BP, localize to regions of active bone metabolism or injury, which include knee and shoulder joints in mice. TCO-BP therefore provides a simple means to assess the effectiveness of new radiolabeled tetrazines to deliver isotopes *in vivo*. Evaluation of the bio-distribution of all five ^{99m}Tc-tetrazines showed uptake in knee and shoulder joints 6 h post injection, demonstrating successful pre-targeting to bone *in vivo* (Figure 4). Previous studies confirmed that radiolabeled TCO-BP accumulates at the bone[18], whereas the ^{99m}Tc-tetrazine construct (**2**) given alone does not (Figure 5). This allows one to conclude that bone uptake was due to the IEDDA reaction.

The more lipophilic constructs **1** and **2** had similar distribution data including high uptake in the knee (9.1 ± 1.9 (**1**); 7.6 ± 2.7 (**2**)) and the shoulder (4.6 ± 1.4 (**1**); 4.8 ± 1.9 (**2**)). High radioactivity concentrations were also seen in the gall bladder, liver and intestines, which is consistent with the distribution of the lipophilic ^{99m}Tc-tetrazine compound **2** in the absence of TCO-BP (Figure 5). Other non-target tissues and organs such as the skeletal muscle and spleen did not show any significant uptake (<1%) when bio-distribution studies were performed on the ^{99m}Tc-tetrazines in the absence of the TCO-BP (Figure 5), so these organs were not taken for the pre-targeting experiments. Additionally, bio-distribution experiments with the ^{99m}Tc-tetrazines alone revealed good clearance from non-target tissues at 6 h post injection. Consequently, this time point, which is within one half-life of the isotope, was selected as the time point for comparing the different radiolabeled tetrazine ligands.

The more polar ^{99m}Tc-tetrazine compound **3** bearing a PEG_5 linker showed very high knee and shoulder uptake (16.2 ± 4.8 and 20.7 ± 4.9 respectively). There was also lower activity observed in the liver and intestines. The corresponding PEG_{10} derivative also showed binding to the bone and reduced uptake in the liver compared to compounds **1** and **2.** The most polar derivative **5**, showed lower bone binding than all other constructs which is likely due to its rapid clearance.

The high bone uptake and bone:blood ratios (Table 1) particularly for compounds **3** and **4** demonstrate that pre-targeting and the IEDDA reaction can be used to localize ^{99m}Tc-labeled compounds *in vivo*. The methods reported here can be used to evaluate any radiolabeled tetrazine including next generation of Tc(I)-tetrazine ligands. It should be noted that for the class of ligands that were used in this study, the structures can be readily varied by changing the nature of the donor groups and linkers between the metal complex and the tetrazine, without significantly altering the ligand synthesis method[21]. Once a lead molecule is identified, an instant kit method, which will likely include solid phase purification methods, can be developed to support clinical translation.

The Tc(I) complexes reported here create the opportunity to prepare new ^{99m}Tc radiopharmaceuticals using a wide array of different TCO-derived targeting molecules including antibodies. Antibodies, despite their excellent targeting properties prior to the creation of technetium labeled tetrazines, would not typically be used with ^{99m}Tc because of their slow clearance (days), which is much longer than the half-life of the isotope (~6 h). An additional application of the chemistry reported here is that the same class of ligands can be prepared with the beta emitting radionuclides ^{186}Re and ^{188}Re. The isostructural Re(I) analogues of the Tc(I) agents when combined with the tumor seeking properties of TCO-BP can be used to treat bone metastases.

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DISCLOSURES:
The authors declare they have no competing financial interests.

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Figure 1 tif

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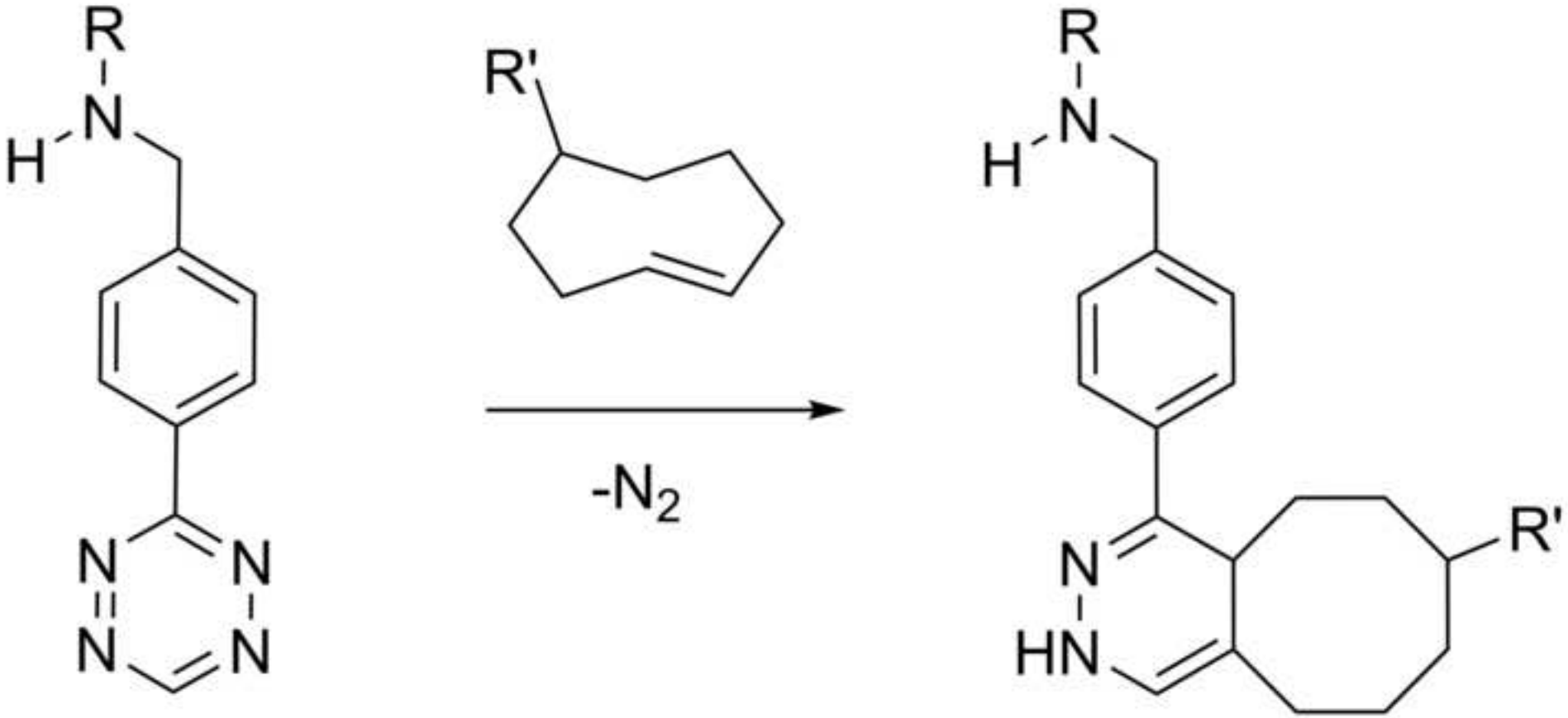

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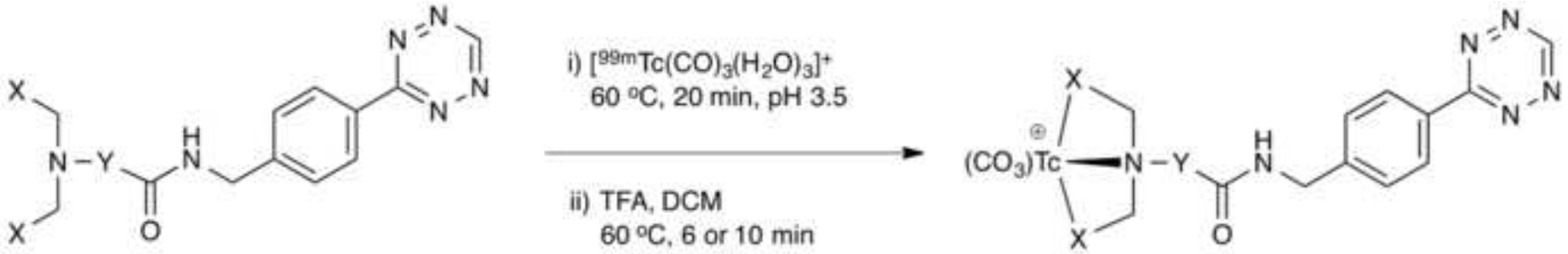
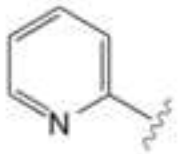
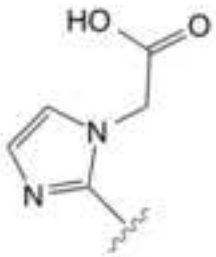
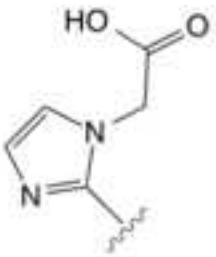
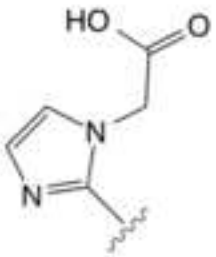
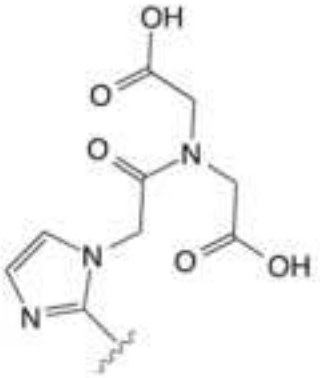
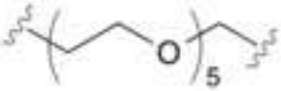
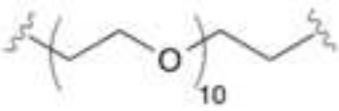
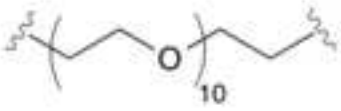


Compound

	1	2	3	4	5
X					
Y					

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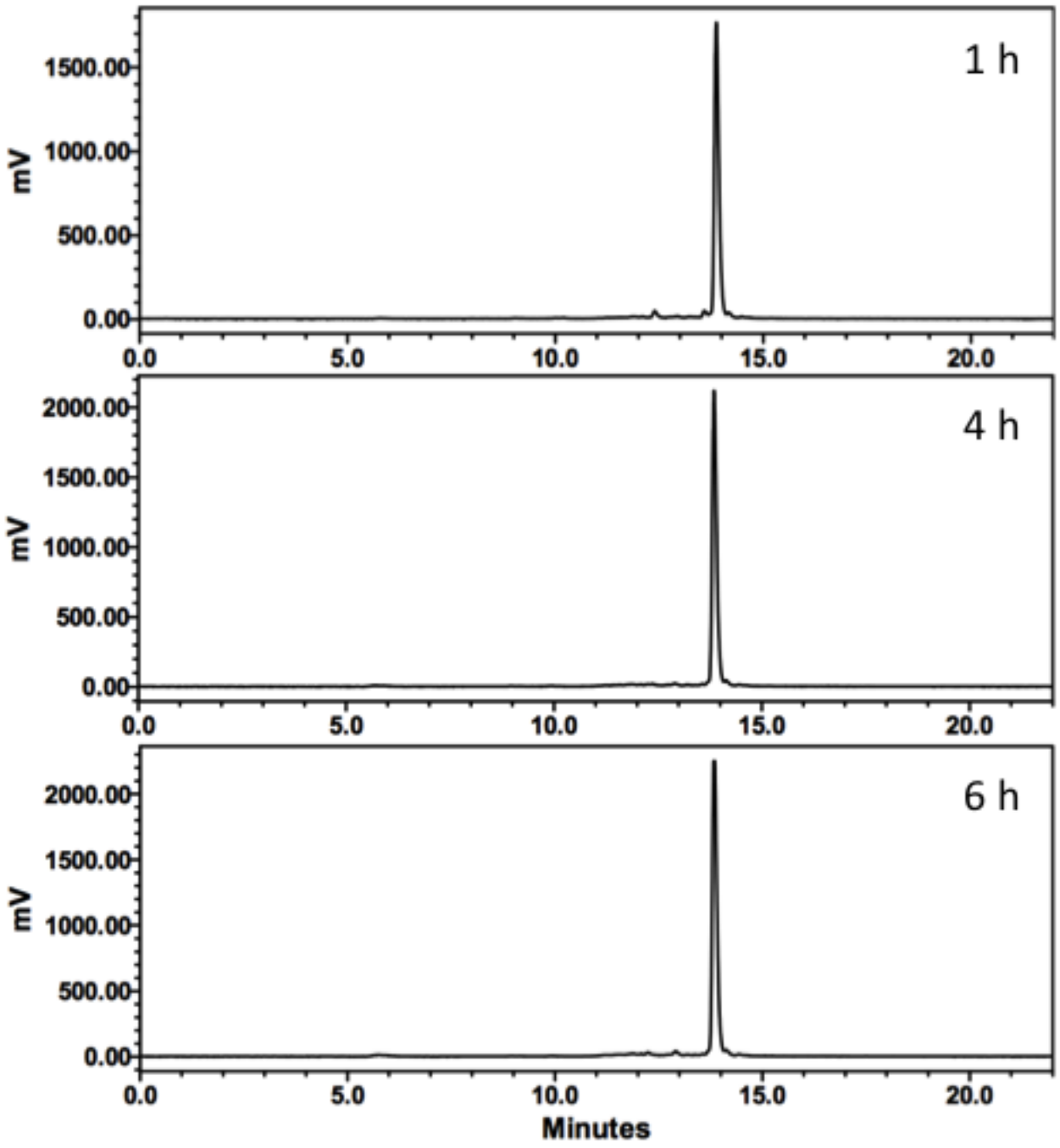

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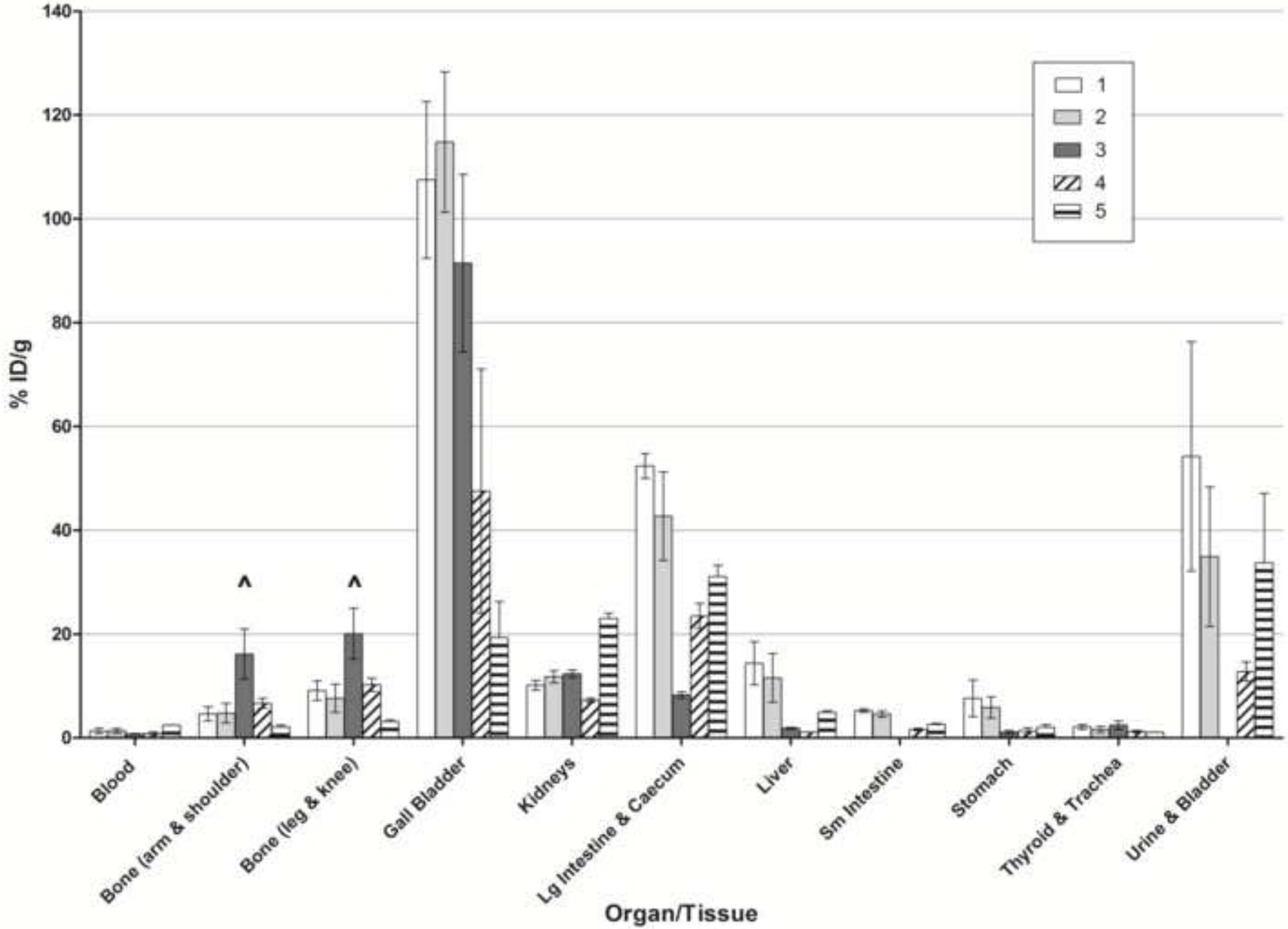

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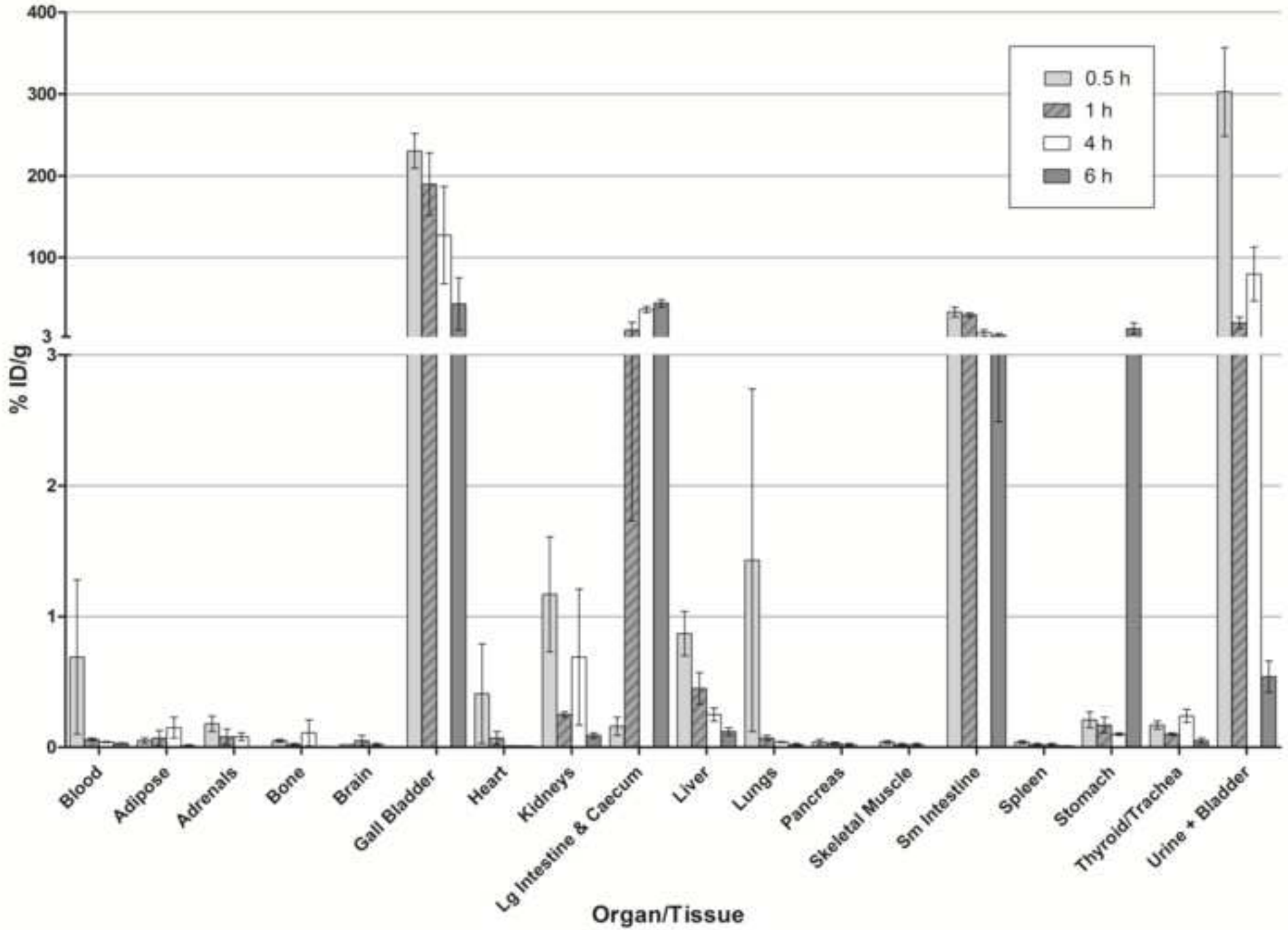

Table 1

	Compound				
Ratio	1	2	3	4	5
Shoulder: Blood	3.5 : 1	3.5 : 1	21 : 1	7.8 : 1	0.8 : 1
Knee: Blood	6.9 : 1	5.6 : 1	26 : 1	12 : 1	1.3 : 1

Excel Spreadsheet- Table of Materials/Equipment

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Name of Material/ Equipment	Company	Catalog Number
Argon gas	Alphagaz	---
Na_2CO_3	EMD Millipore	106395
$Na_2B_4O_7.10H_2O$	Anachemia	S9640
$KNaC_4H_4O_6.4H_2O$	Anachemia	217255
Technelite ^{99m}Tc generator	Lantheus medical imaging	---
0.9% Saline	Lantheus medical imaging	---
1 M HCl	Lab Chem	---
MeOH	Caledon	---
ACN	Caledon	---
Millipore H2O	Thermo Fisher Scientific	Barnstead Nanopure
DCM	Caledon	---
TFA	Caledon	---
PBS	Thermo Fisher Scientific	10010023
BSA	Sigma Aldrich	A7906
Tween80	Sigma Aldrich	P8047
Isoflurane	CDMV	108737
HPLC	Waters	1525 Binary Pump, 2998 Photodiodde Array Detector, E-SAT/IN, Bioscan Flowcount PMT detector (item # 15590)
HPLC column for analysis and purification of compounds 2-4	Phenomenex	00G-4435-E0
HPLC column for analysis and purification of compounds 1 and 5	Waters	186003115
Microwave Reactor	Biotage	Initiator 8
Biotage V10 Evaporator	Biotage	Serial # V1041
Dose calibrator	Capintec, Inc.	CRC-25R
Gamma counter	Perkin Elmer	Wizard 1470 Automatic Gamma Counter
Animal room scale	Mettler Toledo	XP105 Delta Range

Microwave vials	Biotage	355629

Comments/Description
Source of 99mTcO4- To elute generator
HPLC grade
pH 7.4 1X
Supplier: Fresenius Kabi Animal Health
Gemini® 5 µm C18 110 Å, LC Column 250 x 4.6 mm,
XBridge BEH C18 Column, 130 Å, 5 µm, 4.6 mm X 100 mm

0.5-2 mL

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Author(s): Holly A. Bilton, Zainab Ahmad, Nancy Janzen, Shannon Czorny, John F. Valliant

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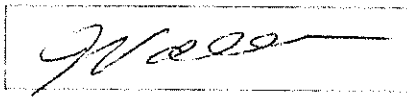
Signature:	
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Response to Reviewers

Responses to Reviewer 1.

1. *Reviewer: P1 l88: give references for tetrazines labelled with 11C, 18F etc.*
   Response: The requested change has been made.

2. *Reviewer: Protocol part 1.2. Why so large amounts (high concentration) of ligands? These ligands are very potent and 100 times less would do it as well. This could save lengthy purification procedures. Comment about this in the discussion section.*
   Response: The high concentration of ligands was needed to maximize the radiochemical yield and reduce reaction times. In the literature, including Ref 21, 10^{-4} M of ligand is typically used for labeling this class of ligands. When the reaction was repeated with the tetrazine ligand at this concentration, radiochemical yields were low and degradation of the tetrazine was observed. As a result, higher concentrations of ligand were used, which significantly improved the radiochemical yield and reduced the synthesis time, and in turn minimized the degradation of the tetrazine. Notwithstanding the presence of additional ligand, the reported purification method is robust and produces the product in high effective specific activity. To ensure clarity for readers, the rationale behind the concentration used and the need for purification were added to the discussion.

3. *Reviewer: What is the chemical reason for steps 1.2.4 to 1.2.6? In step 1.2.3, the compound is formed and then purified from cold material in step 1.2.7. Comment about this.*
   Response*:* The purpose of these chemical reaction steps is to remove the t-butyl protecting groups on the imidazole portion of the ligands. Removing these groups results in carboxylic acid groups, which improves the solubility of the product and enhances the rate of clearance from non-target organs and tissues. This step is performed before the purification of the cold material to avoid the need for two separate purification steps which reduces the overall yield.

4. *Reviewer: Same question for the radiochemical yields after labelling the tetrazines. Why are the yields low? Given the ligand concentration in the labelling solution of about 0.3 mM, quantitative labelling is expected which is the strength of this approach. Side reactions? To underline the strength of this method, the labelling yield should be commented clearly in order to make the approach quantitative.*
   Response: Tetrazines are known to be sensitive to heating and the presence of highly basic groups. Consequently, we suspect that the modest yields are due to decomposition of the tetrazine. In addition, all yields reported are based on the amount of material obtained following HPLC purification where there is inevitably additional loss of material. The radiochemical yield based on HPLC analysis of the reaction mixture is much higher suggesting that the reactions reported are efficient. The yields reported however are isolated yields and are more representative of what one would expect to isolate with the methods reported.

Responses to Reviewer 2.

1. *Reviewer: Since the focus of the journal is on methods rather than on scientific data, it would be more appropriate to change the title by removing the word 'Evaluation' as this would imply some kind of experimental study.*
   Response: The focus of this manuscript is the methodology for both the preparation of the ^{99m}Tc-labeled ligands, as well as the steps taken to evaluate the tetrazines in vivo. We feel that the steps taken to evaluate these ligands are an important part of developing the technology. For this reason, we feel the title accurately represents what is being presented within the manuscript.

2. *Reviewer: For the same reason, it doesn't seem appropriate to include animal studies because these do not contribute to clarify the method. As explained in the Introduction, this paper is aimed at describing a pre-targeting method grounded in the bioothogonal Diels-Alder cycloaddition reaction between tetrazine and trans-cyclooctene (TCO) as a general tool for discovering new target-specific Tc-99m radiopharmaceuticals.*
   Response: We agree with the reviewer that the main focus of this manuscript is the synthesis and labeling of these ligands for a pre-targeting approach. However, we believe that demonstrating successful pre-targeting *in vivo* is a crucial step in validating the utility of any new tetrazine ligand*.*

3. *Reviewer: Thus, to limit its application to targeting bone metabolism falls short in showing its general applicability. In other words, it is supposed that the method must work and there's no need to demonstrate its effectiveness, but simply to precisely describe the full procedure. Actually, using this method to develop another example of bone targeting agent does not seem to stimulate any particular interest, as it would happen with other more attractive biomolecules such as antibodies. Moreover, the whole Tc-99m conjugate, assembled by carrying out the bioortogonal addition in solution before injection, is also expected to show some bone accumulation, thus making the pre-targeting approach unnecessary. To propose this chemistry as a method for developing an entire new class of target-specific Tc-99m radiopharmaceuticals, the whole procedure has to be very precisely outlined and this information should include the most optimal tetrazine derivatives as well as the most effective TCO derivatives, possibly being designed with the most favorable structural characteristics. Notably, this information on the composition of TCO-bisphosphonates was not reported in the paper. Unfortunately, the whole preparation looks very cumbersome, as it requires a remarkable number of steps comprising the use of non-aqueous solvents and evaporation, and a final purification by HPLC. It's hard to see how these complex manipulations could be translated in a clinical setting. The observed radiochemical yields ranged between 31−83%, thus suggesting a high variability of the coordination affinity of the various tetrazine ligands. Highly variable bone accumulation was also found for the different Tc-99m chelates, a fact that seems to indicate that the in vivo bioortogonal reaction does not always occur with the highest efficiency. All these limitations have not been properly addressed into the paper. In summary, to make the outlined procedure more solid and similar to a general-purpose method, some more optimization would be beneficial.*
   Response: The rationale behind using a bisphosphonate as the targeting ligand, as opposed to an antibody, is that it is a more convenient means of evaluating new tetrazines in vivo (lower cost, no need to use expensive tumor models, simpler experimental design, etc.). It was NOT to create a new radiopharmaceutical capable of targeting bone. Once pretargeting using a new labeled tetrazine and the TCO-bisphosphonate has been proven

effective, the new Tc-tetrazine complexes can be evaluated with other TCO-functionalized biomolecules (antibodies, peptides, etc). This approach can be used with any new labeled tetrazine (and to compare existing tetrazines head-to-head) and is therefore generally applicable; much more so than the TCO-derived antibodies used in the literature which are typically not widely available.

The biology clearly shows that each of the labeled tetrazine ligands can react with the TCO-bisphosphonate in vivo. The difference in accumulation of each Tc-ligand at the bone is due to the varying structures and polarities of each tetrazine, which is discussed within the manuscript. As shown between the five ligands, there is an increase in bone uptake from compounds 1-3, followed by a decrease in compounds 3-5 respectively. Therefore, significant effort around optimization of the compounds was conducted and discussed.

One of the key goals of the manuscript was to develop a robust method for producing tetrazines labeled with ^{99m}Tc, so that the utility of pretargeting with the most widely used isotope in diagnostic medicine could be evaluated preclinically. It is premature to develop a single step instant kit production method suitable for clinical use until a lead construct based on a TCO-derived biomolecule is identified. The method used here can be readily used in any radiochemistry laboratory opening the door to using pretargeting (or simply TCO-tetrazine chemistry) to create new technetium radiopharmaceuticals. As for the information on TCO-BP, the synthesis is presented in detail in reference 18. The details for all other procedures from labeling to in vivo testing are provided in the manuscript.

Responses to Reviewer 3.

1. *Reviewer: The appeal of this method is that the animal model is straightforward (normal mice pre-treated with TCO-BP, no need for xenografts) and yet challenging (need for the i.v. injected tetrazines to reach the sites of TCO accumulation, extravasate in high enough concentration, etc.), more than, for instance, the model of "blood pretargeting" used by Devaraj et al. in PNAS (2012). In such model, a side-by-side comparison provides useful information on tetrazine in vivo reactivity towards TCO, pharmacokinetics, and stability combined, in a short time, making a preliminary selection easy. However, a one-time point biodistribution is not enough to characterize the in vivo behavior of a new tetrazine and this preliminary evaluation must be followed by a more thorough in vivo characterization (blood kinetic, clearance from liver and kidney, excretion profile, etc.). For instance, in this study tetrazine 3 appears to be the most promising among the compounds tested. However, at 6 h post-injection compound 3 still has an elevated retention in gallbladder (ca. 90%ID/g) and kidney (> 10%ID/g), significantly higher than other tetrazines in the literature. Most likely such a probe would be not suited for pretargeted radioimmunotherapy, unless it clears effectively from non-target tissues at later time points, which needs to be assessed in a follow-up study. The authors somehow mention the screening nature of their approach in the introduction, but then this concept is lost in the discussion. In my opinion, the preliminary nature of this comparison should be stressed and, possibly, discussed further.*
   Response: The proof of concept nature of the results presented in the manuscript has been highlighted within the discussion section. The intention was to develop a method to create a new class of Tc(I)-tetrazine ligands and to show how a TCO-derived bisphosphonate could allow for simple, cost-effective and rapid screening of these new complexes in vivo. We feel it would be beyond the scope of a JOVE article and of lesser interest to readers to do an

extended time course study on each of the ligands. For clarity, the reasoning as to why the 6 h time point was chosen was added to the discussion section.

2. *Reviewer: One major concern is that in this study the authors show the biodistribution of 5 different tetrazines in mice pre-treated with TCO-BP and they conclude that the activity accumulation in joints proves tetrazine reaction with TCO in vivo. However, without negative controls one can only speculate on the (reasonably) TCO-specific tetrazine accumulation in joints. Especially in view of the scope of JoVE (the dissemination of scientific methods), the authors should present also tetrazine biodistribution in mice that were not pre-treated with TCO-BP or that were pre-treated only with bisphosphonates. Apparently biodistribution studies with tetrazines in the absence of TCO-BP were carried out by the authors but the data are not shown in this manuscript. In my opinion, this is key information to support any conclusion. In case the complete dataset is presented in the manuscript of Ref. 13 (submitted but not yet accepted), the authors should still give enough information here (or they should wait for acceptance of Ref. 13).*
   Response: A figure containing control biodistribution data of the labelled tetrazine compound alone (no TCO-BP administered to the animal) was added to the manuscript. The data showed no uptake in bone.

3. *Reviewer: The authors should report the specific activity of the 99mTc-tetrazines they injected in mice (or the moles they have injected). They report the amount of precursor used for radiolabeling (2 mg for all tetrazines, although their MWs are quite different) but then they do not disclose whether the excess (unlabeled) tetrazine was separated from the labeled ones during post-labeling HPLC purification. The administration of different amounts of tetrazines, if this is the case here, may be the reason for different uptake in joints, especially in the presence of a limited amount of TCO.*
   Response: The specific activity for the Tc-labeled Tz constructs has been added as requested. In addition, a comment regarding the ability of the reported purification method to remove unlabeled ligand was added to the manuscript.

4. *Reviewer: In the long abstract the author state that the 99mTc-tetrazines are stable in saline and PBS but then do not report stability studies. These experiments should be shown or a suitable reference should be given.*
   Response: A protocol for assessing stability, and a figure showing stability results has been added to the manuscript.

5. *Reviewer: In the introduction the authors should state the solvent that was used to determine the k2 value they report as K2 changes enormously in, for instance, methanol vs water.*
   Response: The solvent that was used has been added to manuscript.

6. *Reviewer: In the protocol section (1.2.8) after HPLC purification the authors evaporate the solvent with a rotavapor. To my knowledge the gold standard in radiotracer synthesis is solid phase extraction to eliminate all traces of MeCN and TFA before formulation. Is there a reason why this method was not applied to the tetrazines in this study?*
   Response: A V10 evaporator was used to evaporate HPLC solvent to save time during the labeling procedure rather than conducting solid phase extraction to remove MeCN and TFA. The latter can be done in the absence of a V10 evaporator but requires additional time.

7. *Reviewer: In the protocol section (2.2.9) the method that was used to calculate the %ID is not clear. To my knowledge, the gold standard is to pre/post-weigh syringes then weigh and count aliquots of the injected dose along with the tissue samples (method that does not require further decay correction when using modern gamma counters). Here I don't understand if all organs and tissues (including carcasses), and possibly the cage bedding, etc., were counted in order to estimate the injected dose. The authors should better explain what they did.*
   Response: The method the reviewer has suggested was similar to the one used to generate the data in the manuscript. Additional detail on the biodistribution protocols including the method by which %ID/g was calculated, and the use of standards for the gamma counter, have been added to the protocol section.

8. *Reviewer: In Fig 3 the authors don't show the tetrazine biodistribution in important non-target tissues (e.g. muscle and spleen). If available, these organs should be added to the figure for completeness of information.*
   Response: In a separate biodistribution study where a full organ list was used, there was no significant uptake in the muscle or spleen, along with other non-target organs. Therefore these organs were not taken during the reported biodistribution experiment. This explanation was added to the discussion section, as well as a figure of a healthy mouse biodistribution study to justify the decision. The key was to compare the extent of uptake in the shoulder and knee joints between the pre-targeted and control experiment, which is an indicator of whether or not the labeled tetrazine is able to combine with the TCO-bisphosphonate in vivo.

9. *Reviewer: Also in Fig. 3 the authors should replace the "*" used to indicate the bones with a different symbol as it is confusing (usually this symbol denotes statistical differences).*
   Response: The "*" symbol has been changed to the symbol "^" in the figure (now Fig. 4) and in the figure legend.

Response to Reviewer 4.

1. *Reviewer: This manuscript describes the methodology very well and is very helpful for the scientists who are using this method. No serious problem was found in this manuscript.*
   Response: We thank the reviewer for the positive comments.

2. *Reviewer: The authors indicated the place where the Figure 1 should be located, which is good. However, they didn't indicate the places for other Figures, which should be indicated.*
   Response: Approximate placing for figures 2-5 were added.

3. *Reviewer: Figure 2 and 3 are not seen, which looks like due to a technical problem of the on-line submission software.*
   Response: This issue seems to be specific to this reviewer as the other reviewers did not have this problem. All figure files that were part of the resubmission have been submitted as .tif files and their quality verified.