Research Project Report

Marshall Plan Fellowship

Bioorthogonal Chemistry - Inverse Electron Demanding Diels Alder Reaction

Rapid Radiolabelling Using Fast Tetrazine Ligation

Conducted by
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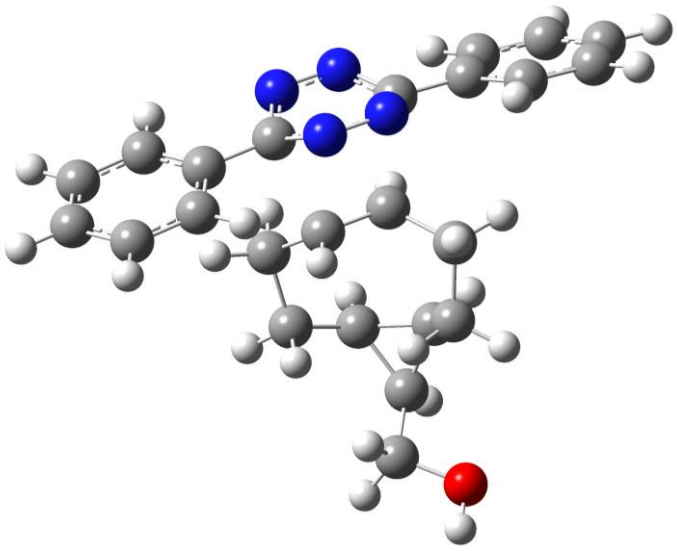

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Abbreviations

Cis-sTCO	(rel-1R,8S,9S,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol
dTCO	((3aR,9aS,Z)-3a,4,5,8,9,9a-Hexahydrocycloocta[d][1,3]dioxol-2-yl)methanol
FMO	**F**rontier **M**olecular **O**rbital
HOMO	**H**ighest **O**ccupied **M**olecular **O**rbital
LUMO	**L**owest **U**noccupied **M**olecular **O**rbital
PDR	Pull-down reagent
PEG	poly ethylene glycol
PET	Positron Emission Tomography
RGD	c(RGDyK), cyclic Arginylglycylaspartic acid derivative
sTCO	(rel-1R,8S,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol
TCO	trans-Cyclooctene
tosyl	p-toluenesulfonate
Trans-sTCO	(rel-1R,8S,9R,4E)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol

Introduction and Motivation

Bioorthogonal ligations refer to reactions which selectively form covalent bonds even in high complex environments like living organisms. To qualify as such a reaction the reactants have to be non-toxic, stable, show a very high selectivity towards each other and the reaction product has to be stable and non-toxic as well.

Since their first introduction in 2000 several bioorthogonal ligations were developed and used in different applications. A critical characteristic of bioorthogonal reactions is the reaction rate. Due to the very low concentrations usually used in biologic media a high reaction rate is beneficial to achieve high conversion within a reasonable timescale. Figure 1 shows the correlation between the second order rate constant of a reaction with both reagents at 1 µM starting concentration and first half life (50% conversion) as well as the approximate time for full (99%) conversion.

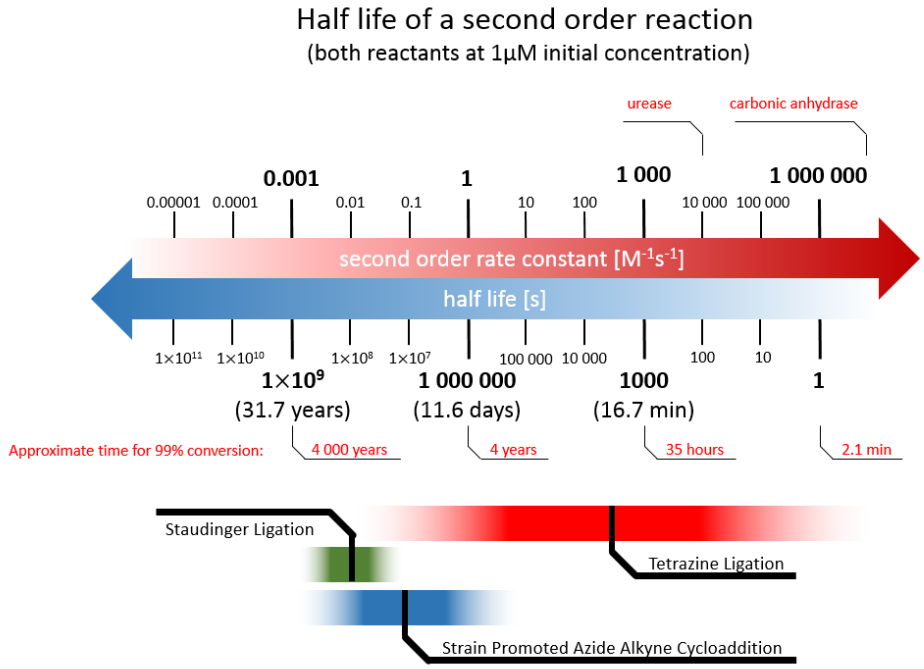
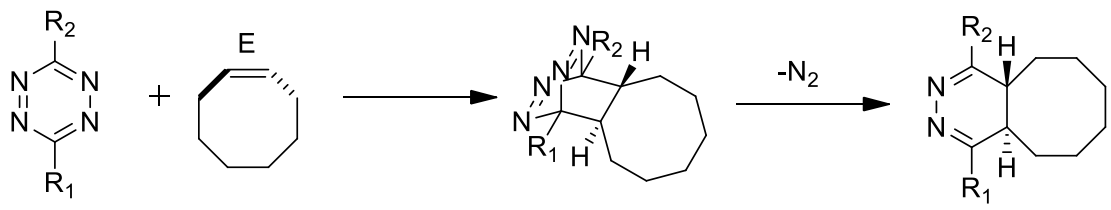


Figure 1: Correlation between second order rate constant and half-life for a reaction starting with equimolar concentration of reagents at 1µM. Approximated reaction rates of selected bioorthogonal reactions are shown at the bottom.

The Tetrazine Ligation

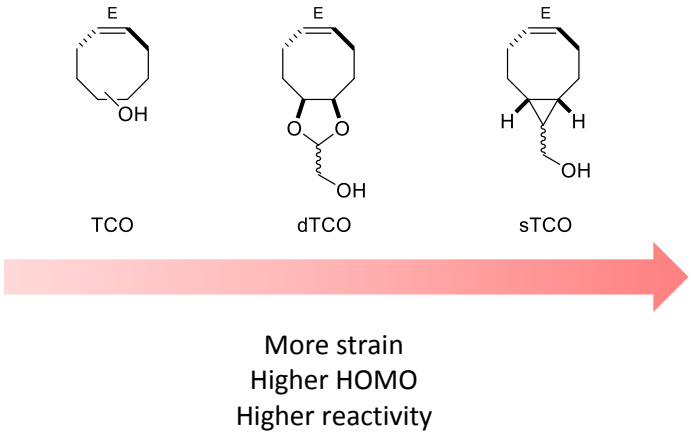
In 2008 Fox et al.[1] and Weissleder et al.[2] independently introduced the reaction between 1,2,4,5-tetrazines and alkenes as bioorthogonal ligation. This two-step reaction starts with an inverse electron demand Diels-Alder reaction between the tetrazine and the dienophile followed by retro Diels-Alder reaction under elimination of nitrogen yielding a pyridazin derivative (Scheme 1), thus linking both reaction partners covalently.



Scheme 1: Inverse electron demand Diels-Alder reaction initiated ligation between 1,2,4,5-tetrazines and trans-cyclooctenes

The big advantage of the tetrazine ligation is the high reaction rates which can be achieved by using the right combination of tetrazines and dienophiles.

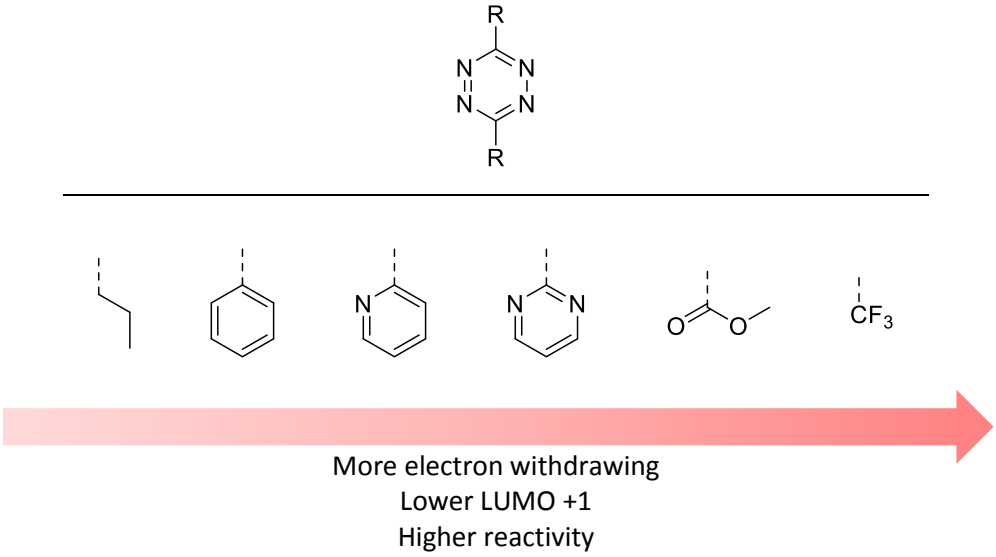
Strained dienophiles like trans-cyclooctenes (TCOs) show exceptional high reaction rates.[3] Fox et al. could show that modifying the backbone of TCO with fused rings leads to higher strain resulting in a higher HOMO and reactivity in the inverse electron demand Diels-Alder reaction. Scheme 2 shows different TCO derivatives. dTCO and sTCO were developed by Fox et al..[4,5] sTCO shows higher reactivity but is more hydrophobic and storage is problematic. dTCO shows lower reactivity but higher water soluble and is shelf stable at room temperature.



Scheme 2: reactivity trend for different TCO derivatives

sTCO was introduced by the Fox group in 2011[5] and is a commonly used dienophile for tetrazine ligation. The higher reactivity in comparison to TCO alcohols comes from a conformational change away from the crown formation to a half-chair formation (Scheme X). As Fox could show the conformational change elevates the HOMO FMO and therefore accelerates the reaction. By using a cis-fused cyclopropene the molecule can be forced into this conformation, while a trans-fused cyclopropene would not cause this conformational change.

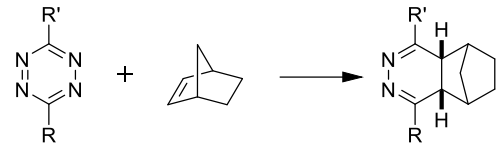
1,2,4,5-Tetrazine reactivity can be influenced by residues on 3 and 6 position. Substituents which lower the low lying empty orbital which is involved in the inverse electron demand Diels-Alder reaction (typically LUMO+1) accelerate reaction rates in most cases.[6] (Scheme 3)



Scheme 3: reactivity trend for different tetrazine derivatives

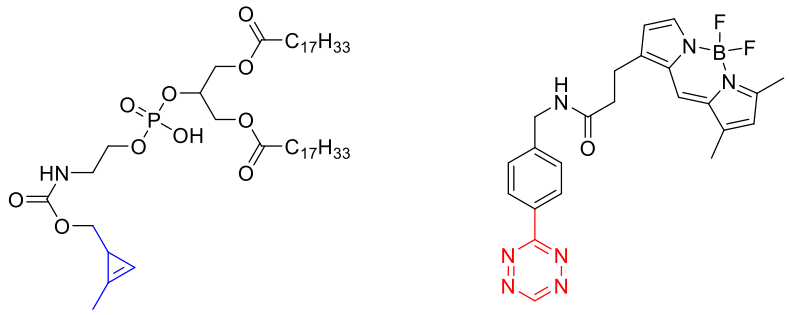
This rate acceleration comes with a drawback. While dialkyl tetrazines are very stable, even in biologic media, 2-pyridyl and 2-pyrimidyl substituted ones show high degradation after a time of 10h.[7] Methylester and trifluoromethyl substituted tetrazines are not stable in aqueous media.

While trans-Cyclooctenes provide exceptional high reaction rates other dienophiles can be used which might have advantages over trans-Cyclooctenes. Norbornenes react quite slowly but are commercially available and stable. In fact, while Fox et al. used trans-Cyclooctenes for their introduction of the tetrazine ligation as bioorthogonal reaction in 2008[1] Weissleder et al. used the reaction of norbornenes with tetrazines in their first paper within this field.[2] Knall et al. evaluated a series of norbornenes regarding their reactivity and reported second order rate constants of their reaction with di-3,6-(2-pyridyl)-1,2,4,5-tetrazine in methanol of about 0.001 to 0.155 $M^{-1}s^{-1}$.[8] Norbornenes were used in site specific labelling of proteins using genetically encoded tagging of aminoacids[9] as well as in polymer functionalization where low reaction rates are not a limiting factor.[10]



Scheme 4: Reaction between norbornene and tetrazines

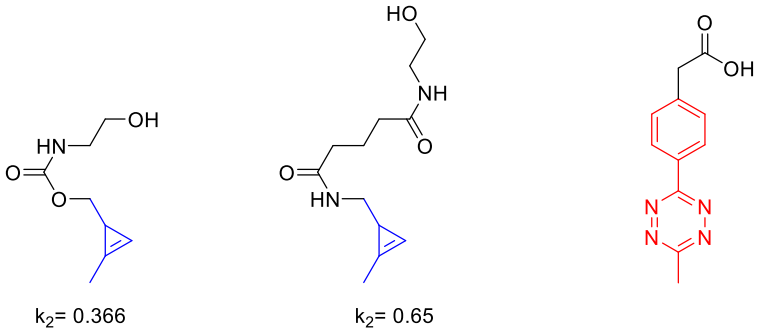
Other common dienophiles are cyclopropenes. In 2012 Devaraj and coworkers introduced the use of cyclopropene derivatives as dienophiles for tetrazine ligation. Based on the work of Sauer et al., who reported that the unstable cyclopropene and 3-methylcyclopropene have high reaction rates with highly reactive tetrazines[11], they were able to develop cyclopropene derivatives with sufficient stability while still obtaining reasonable high reaction rates of up to 13 $M^{-1}s^{-1}$ (12% DMSO in water, 37°C) by using 1-methyl substituted cyclopropenes.[12] They were able to use this cyclopropenes for tagging phospholipides for live cell labeling in combination with a fluorogenic dye containing a tetrazine for ligation (shown in scheme 5).



cyclopropene tagged phospholipide fluorogenic tetrazine probe

Scheme 5: First used system for cyclopropene tetrazine ligation

Cyclopropenes have the big advantage of being very small tags for tetrazine ligation, thus having a minimal steric impact and effect on pharmacokinetic and pharmacodynamics of the tagged molecule. Therefore they can be used in metabolic labeling, since they are most likely tolerated by a wider variety of biologic pathways.[13] Unfortunately the reaction rates are not as high as for other dienophiles like trans-Cyclooctenes. Devaraj et al. noticed that the nature of the substituent in 3 position of the cyclopropene has a large impact on the reactivity as well as the stability in aqueous media of the tag. They were able to nearly double the reaction rate of this ligation by changing the substituent from a carbamat based linker to an amide. (Scheme 6)[14]



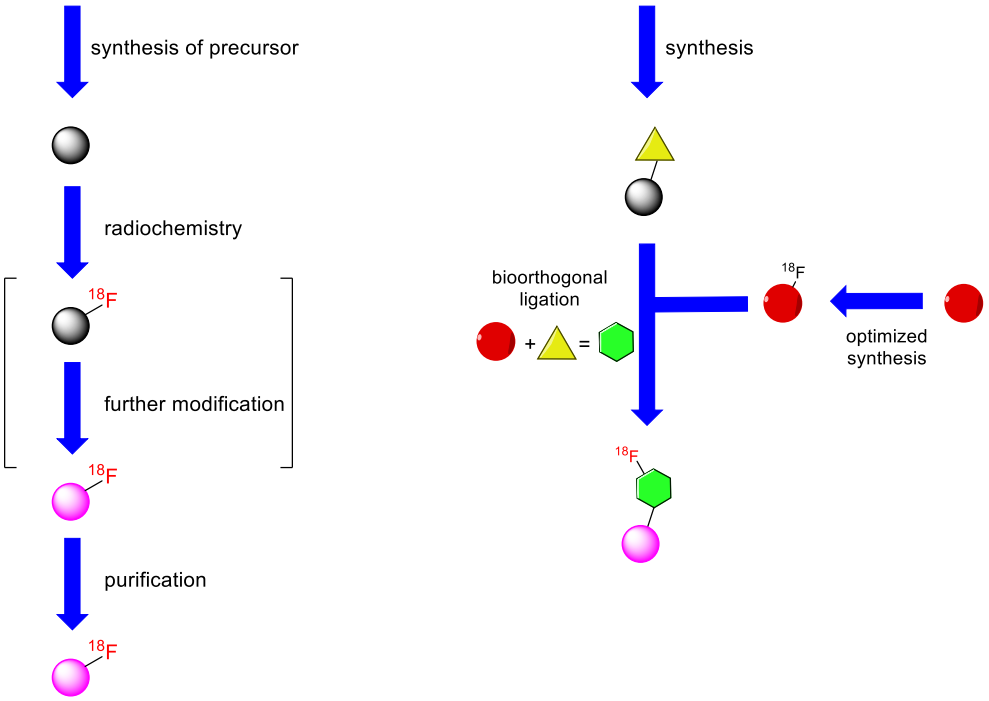
Scheme 6: First generation cyclopropene tag on the left, second generation in the middle. Reaction rates reported for the ligation with the tetrazine on the right in 50 mM MOPS buffer, pH = 7.5, 250 mM NaCl, RT.

Bioorthogonal reactions in radiochemistry

Due to their fast reaction kinetics as well as their nature to be carried out biologic environment biorthogonal ligations can be used in two ways in the field of radiochemistry, especially in the field of PET imaging where short living radionuclides are common.

Rapid Radiolabeling

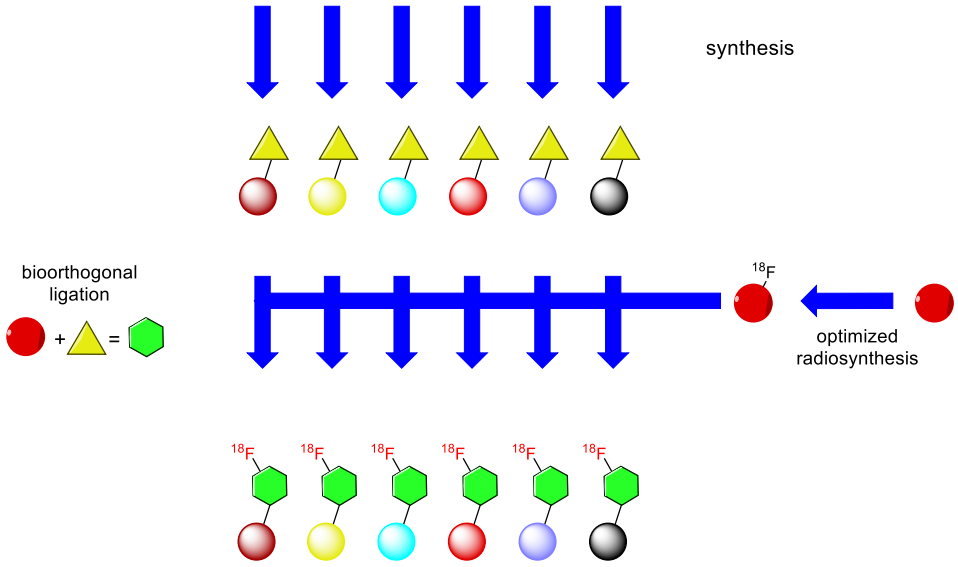
Currently radiosynthesis is a limiting factor in the field of PET. Radiolabelling, especially with short living radionuclides like ^{18}F, the most commonly used radionuclide, is often a challenge. Short half-live as well as poor nucleophility of ^{18}F-fluoride leads to difficult incorporation of ^{18}F in complex molecules. Bioorthogonal chemistry can be used to simplify the synthesis involved in radiolabelling of even complex or large molecules, like antibodies. By introducing one biorthogonal reaction site on the target molecule it is possible to radiolabel this compound by bioorthogonal ligation with its radiolabelled counterpart. This results in fast radiolabelling with no more modification of the compound necessary. (Scheme 7)



Scheme 7: conventional radiolabelling (left); rapid radiolabelling using biorthogonal reaction (right)

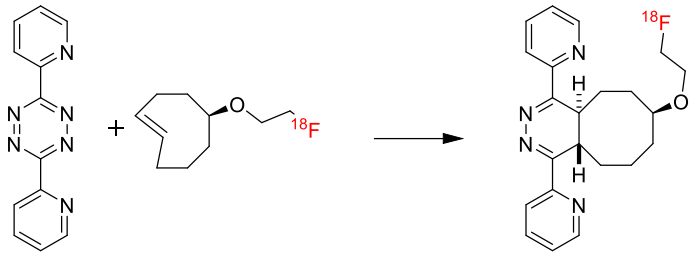
Fast reaction kinetics enables the use of only a small excess of target molecule, resulting in high specific activity.[15] This is particularly interesting for radiolabelling of antibodies, since separation of

radiolabelled and non-radiolabelled compounds might not be possible. Another big advantage is the possibility of radiolabelling a lot of different molecules without optimization of radiochemistry. (Scheme 8)



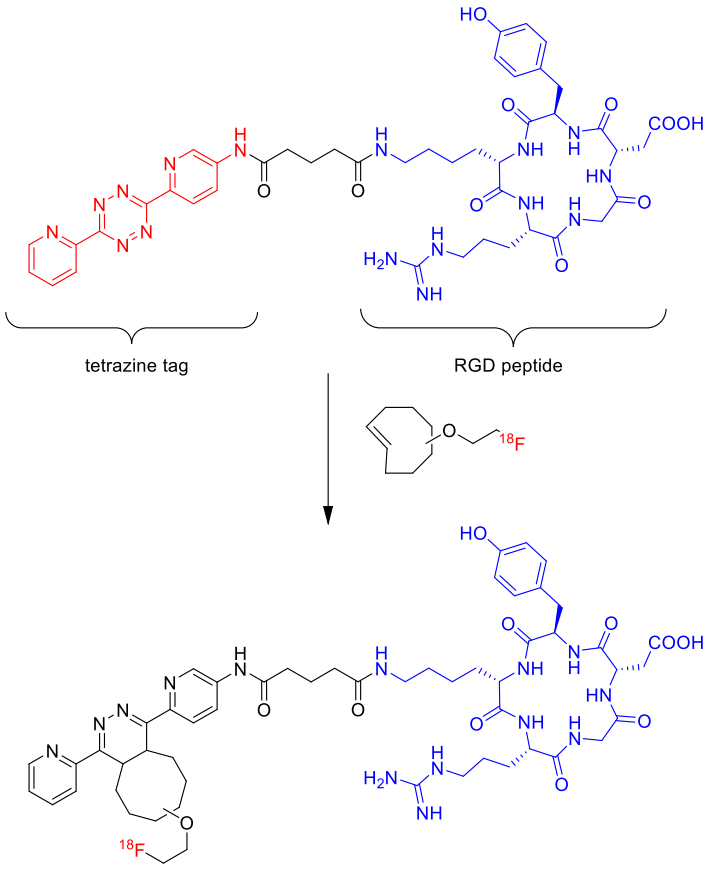
Scheme 8: high throughput radiolabelling using bioorthogonal ligations

The group of Prof. Fox has done a lot of work within the topic of rapid radiolabelling using tetrazine ligation. In 2010 they introduced the rapid construction of ^{18}F labeled probes (Scheme 9) using the TCO tetrazine reaction which set the basis for future work.



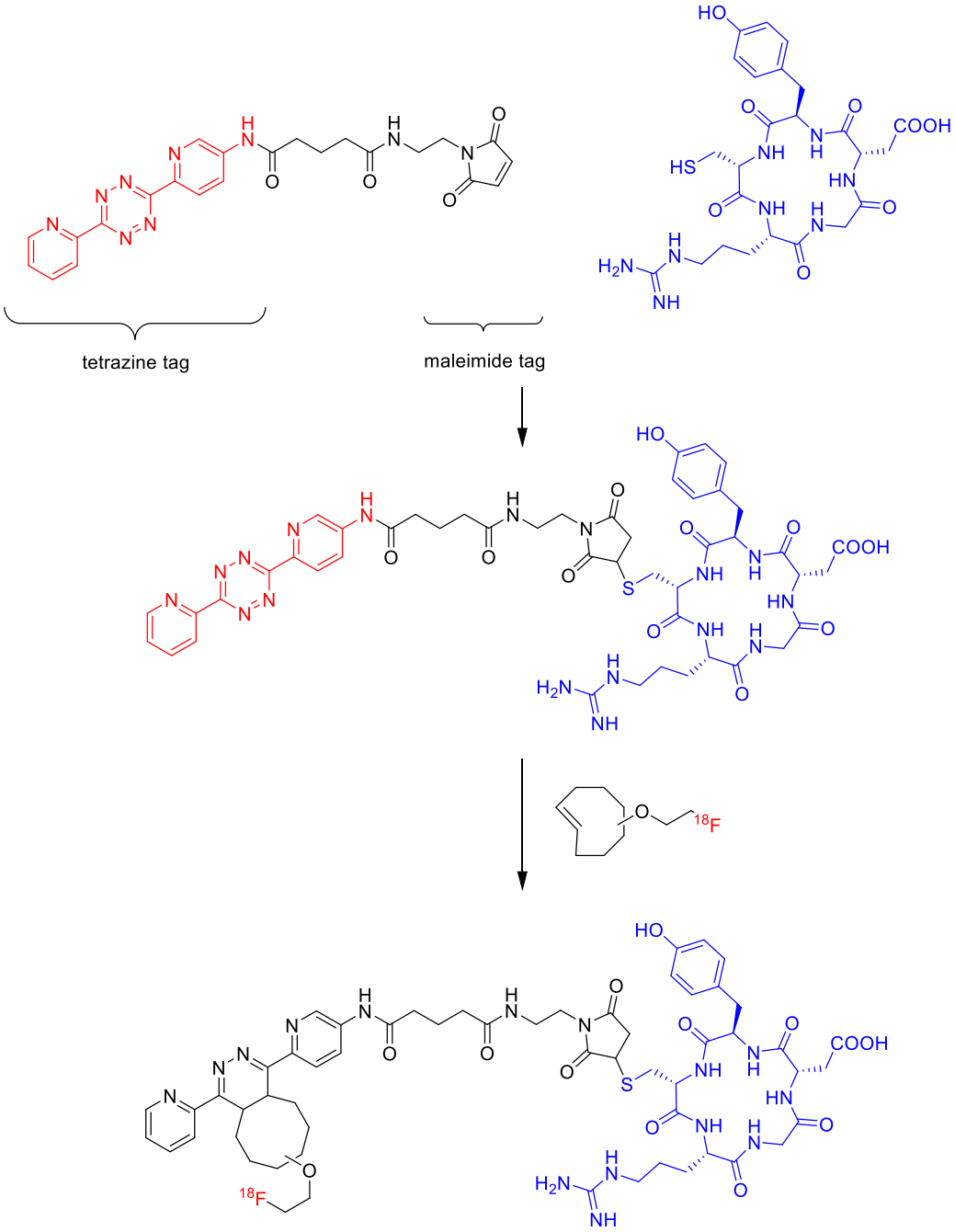
Scheme 9: First work in the field of rapid construction of radiolabeled probes using the tetrazine reaction

In 2011 Fox et al. reported the use of the tetrazine ligation in the rapid radiolabeling of an integrin $\alpha_v\beta_3$ targeted PET tracer based on a cyclic RGD peptide.(Scheme 10)[16] They were able to radiolabel the tetrazine tagged RGD peptide using only 16 equivalents of peptide with a conversion of >90% within 5 min.

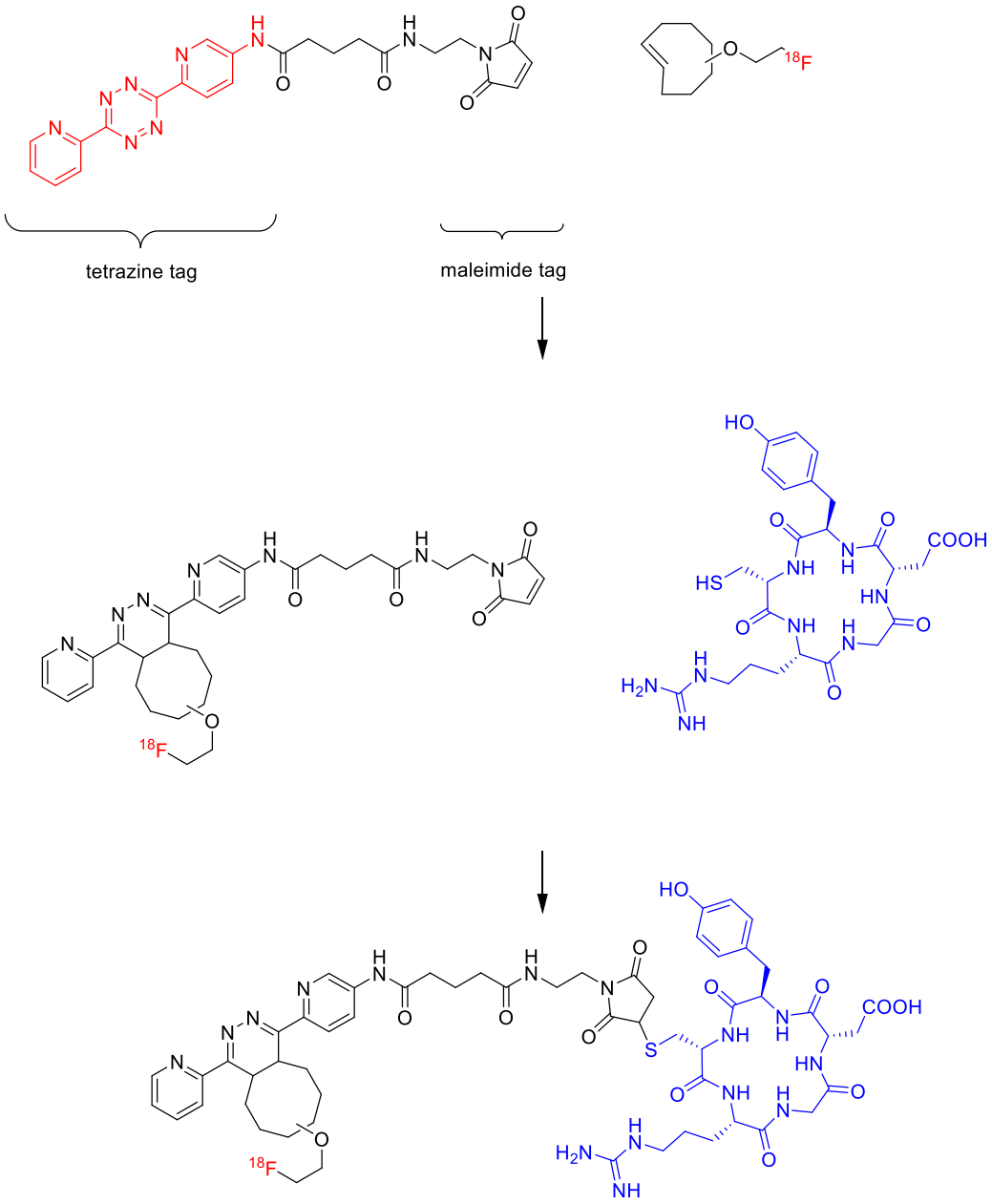


Scheme 10: First rapid radiolabeling of a peptide using the tetrazine reaction

In 2013 they published a method for rapid radiolabeling of cysteine-containing peptides and proteins, using a maleimid modified tetrazine for coupling onto cysteine.[17] After tagging of the peptide/protein with tetrazine rapid radiolabeling could be applied. They were able to demonstrate the power of this approach by radiolabeling of the peptide c(RGDyC) and the protein (VEGFH)-SH. Both could be radiolabeled within minutes with high yields of 95% and 75% respectively. There are two possible strategies with this approach. The tetrazine can first be coupled to the peptide or protein using maleimide coupling and rapid radiolabeling using the tetrazine reaction follows in a second step (Scheme 11) or the maleimide tag can be radiolabeled by tetrazine ligation and afterwards conjugated to a peptide or protein. (Scheme 2) Approach 1 has big advantages over the latter one, since reaction time of radiolabeled compounds is shorter and a higher specific activity could be reached.



Scheme 11: First strategy of radiolabeling RGD peptide using a tetrazine modified maleimide tag

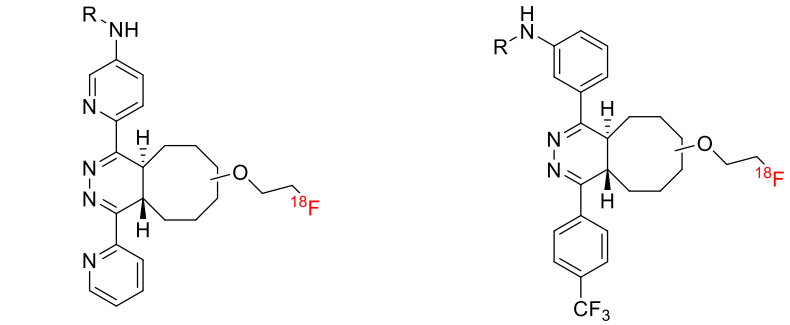


Scheme 12: Second strategy of radiolabeling RGD peptide using a tetrazine modified maleimide tag

Also in 2013 they demonstrated the rapid radiolabeling of an exendin-4 PET probe using the technique described before.[17] This PET probe can be used for monitoring islets. The structure of these probes are shown in Scheme 13.

Scheme 13: First rapid radiolabeling of a peptide using the tetrazine reaction

Recently Fox et al. could show an improvement to their previous used system.[15] The exchange of di-(2-pyridyl) substituted tetrazines against a para-CF_3-phenyl substituted tetrazine leads to a less electrophilic dihydropyridazine core which drastically improves the stability. (Scheme 14)



Scheme 14: Different generation ligation products used by Fox et al.

Pretargeted PET imaging

Due to the short half-life of positron emitters (e.g. ^{18}F), latency between radiotracer injection and measurement of the patient is limited to a few hours. Slow biodistribution, trapping and clearance of PET tracers leads to reduced contrast during PET imaging. To solve the problem of slow kinetics of PET tracers in relation to the short half-life of ^{18}F, a possible solution is the pretargeted PET imaging approach. This means splitting of the tracing compound into a marker, which is trapped in target tissue and a radiolabeled pull down reagent (PDR). By using such an approach, longer periods of time for enrichment of the tracing compound can be provided, which is crucial for PET measurements with high contrast of target tissue to normal tissue. After this labeling the pull down reagent is injected, which should react with the trapped marker in a bioorthogonal way. Figure 2 shows a schematic of such a pretargeting approach.

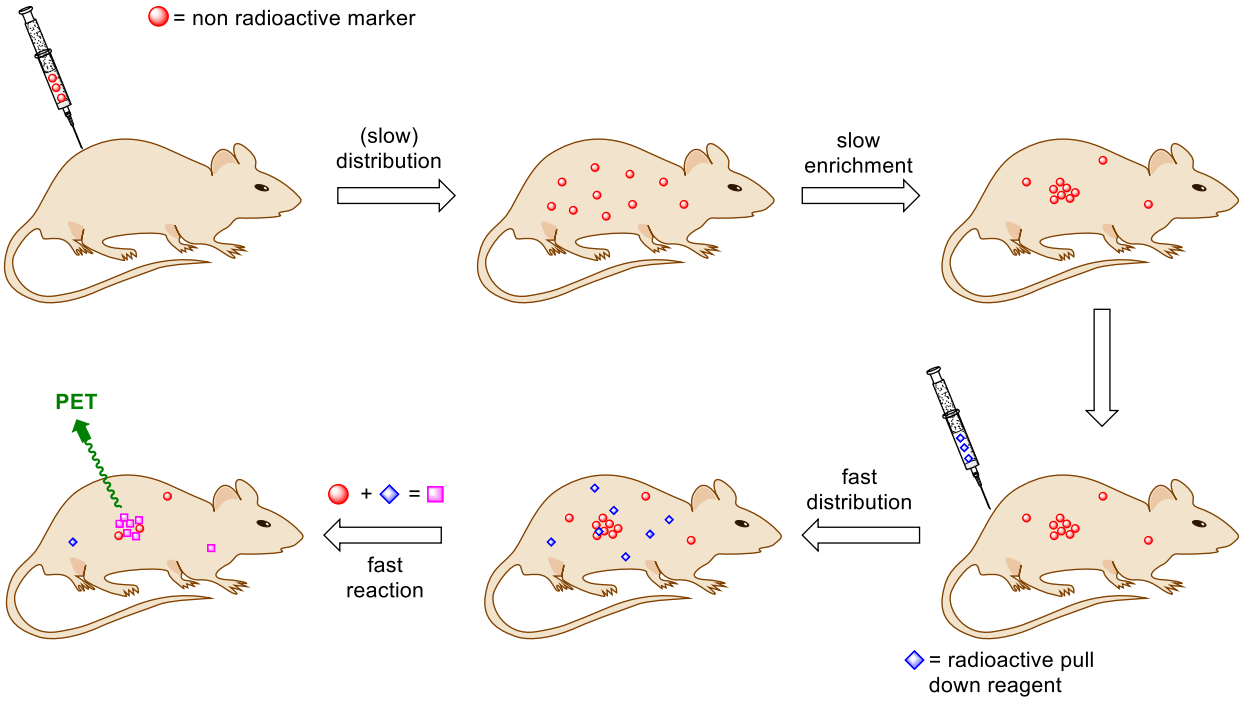
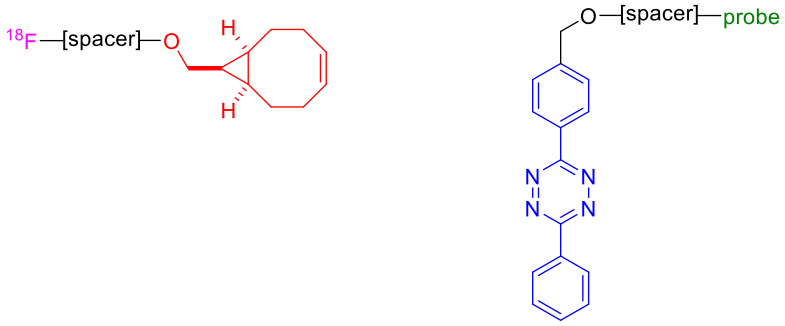


Figure 2: pretargeted PET imaging using a bioorthogonal ligation

Project Report

Aim of project

Aim of this project was the development of a technique for rapid ^{18}F radiolabeling using the tetrazine ligation between sTCO and a diaryltetrazine. This system was developed for radiolabelling of a cyclic Arginylglycylaspartic acid (RGD) peptide but can potentially applied to rapid radiolabelling of antibodies as well as pretargeted PET imaging of suited targets. Scheme 15 shows the target structures of this work.



Scheme 15: target structures

sTCO was chosen due to superior reaction kinetics in comparison to previous used trans-cyclooctenoles.[15,18] A spacer is needed to provide a good handle for radiolabelling. Diphenyltetrazine was chosen as tetrazine in this system due to higher stability in comparison to commonly used di-2-pyridyl substituted tetrazines, a spacer between the tetrazine and the probe is needed to ensure less influence on the probe as well as modification of the biodistribution profile of the radiolabelled probe.

^{18}F labelled sTCO

The target structure is shown in figure 3. It features a sTCO moiety (red) for tetrazine ligation as well as a short PEG linker (purple) and a tosyl group (blue) as leaving group for nucleophilic introduction of ^{18}F via ^{18}F-fluoride.

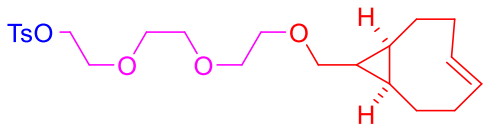


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Figure 3: chosen sTCO precursor structure

There are two different sTCOs as shown in figure 4A. Structure A (anti-sTCO) was described by Fox et al. in 2011 [5] and is the commonly used sTCO diasteromer. Structure B (cis-sTCO) is unknown to literature.

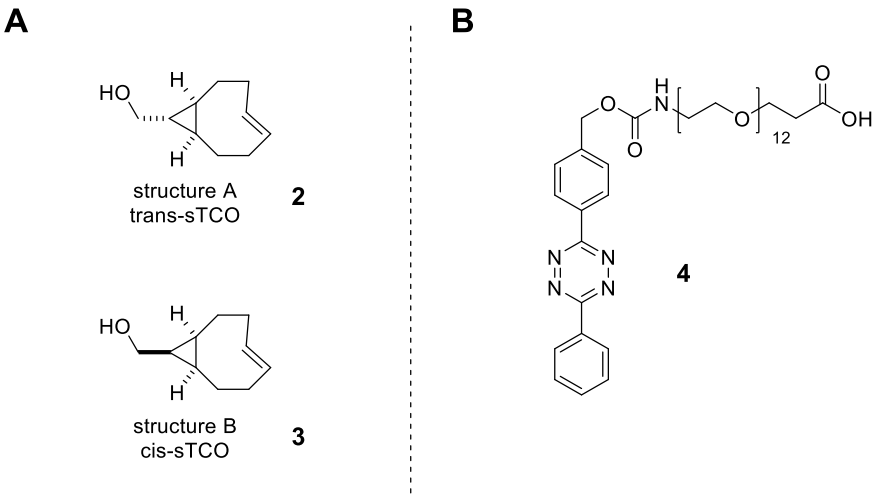
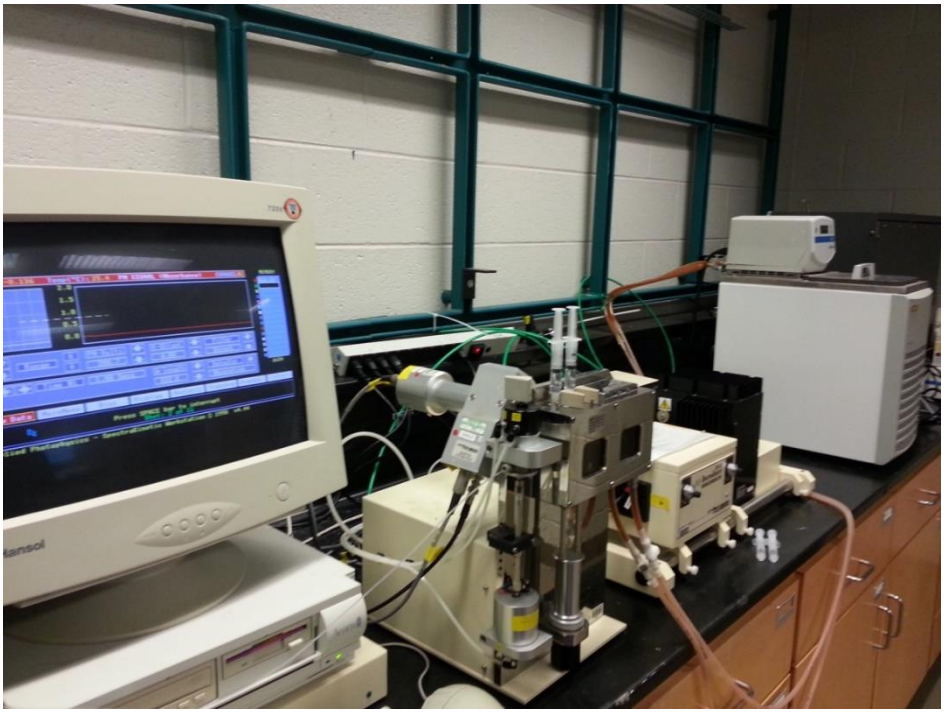
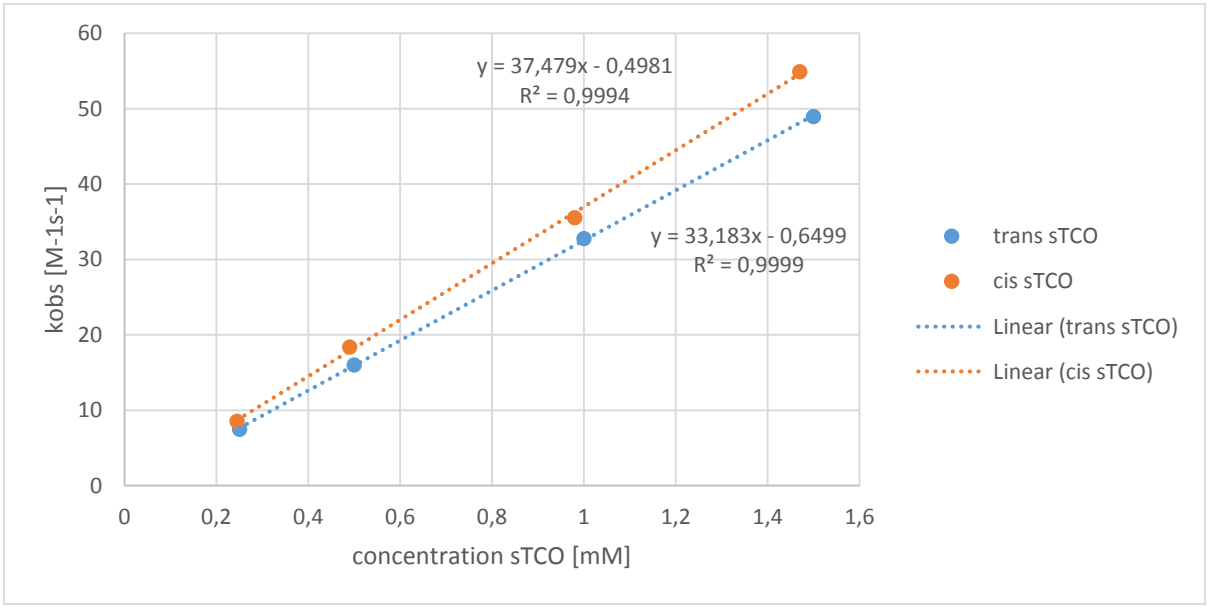


Figure 4: A: possible sTCO structures; B: used tetrazine for kinetic measurements

Part of this work was the evaluation of cis-sTCO for tetrazine ligation. Therefore kinetic measurements of both sTCOs with diphenyltetrazine **4** (figure 4B) were conducted. Measurements were done under pseudo-first order conditions (excess of sTCO) in water:methanol 45:55 by following the exponential decay of the tetrazine at 298 nm over time using an SX 18MV-R stoppedflow spectrophotometer (Applied Photophysics Ltd., picture 1). Solutions were prepared for the sTCO concentrations of about 0.5, 1.0, 1.5 and 3.0 mM and the tetrazine (0.1 mM in water:methanol 45:55) and thermostatted in the syringes of the spectrophotometer before measuring. An equal volume of each was mixed by the stopped flow device. 400 data points were recorded over a period of 1 seconds, and performed in sextuplicate at 298 K. The k_{obs} was determined by nonlinear regression analysis of the data points using Prism software (v. 6.00, GraphPad Software Inc.). Graph 1 shows the correlation between observed k-values and sTCO concentrations resulting in k2-values of 37500 $M^{-1}s^{-1}$ for cis-sTCO and 33200 $M^{-1}s^{-1}$ for trans-sTCO.



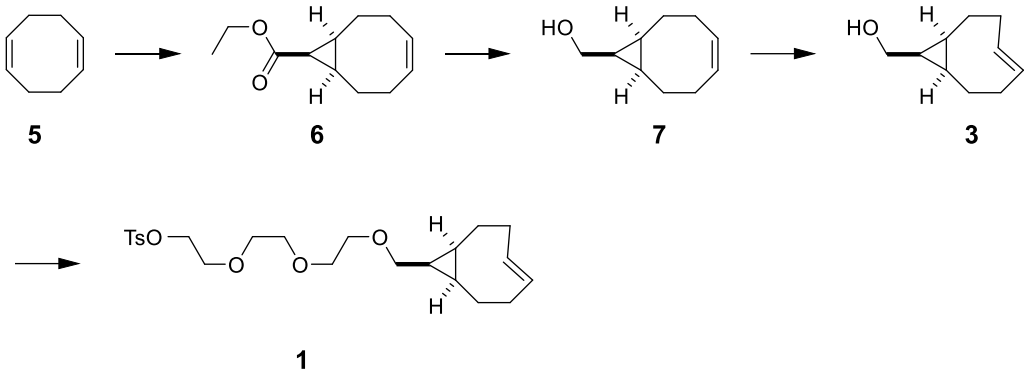
Picture 1: stopped-flow system



Graph 1: observed k-values in relation to sTCO concentration

Cis-sTCO shows an about 14% higher reactivity than trans-sTCO and is therefore be suited for rapid radiolabelling.

Synthesis of sTCO precursor for radiolabelling was done by Dr. Yu Liu (research group of Prof. Fox) resulting in following synthesis shown in scheme 16. This synthesis was repeated by the author of this work to produce needed amounts of radio precursor.

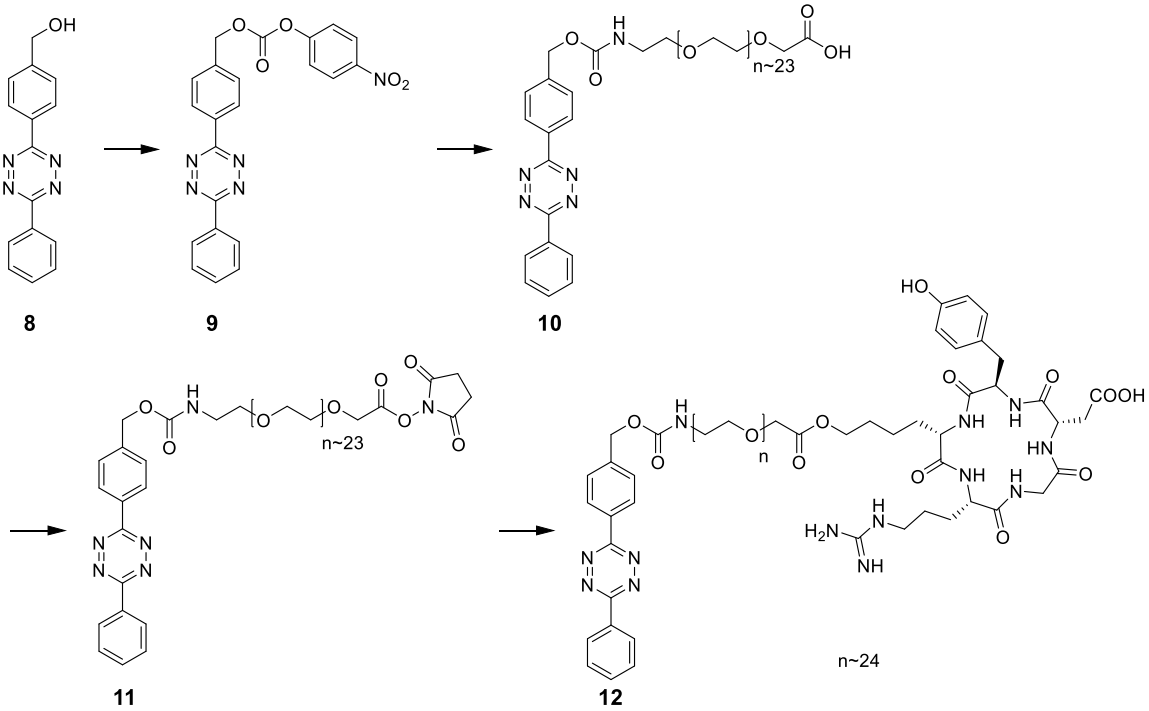


Scheme 16: synthesis of precursor **1**

Starting from 1,5-cyclooctadiene Ethyl (1R,8S,9S,4Z)-bicyclo[6.1.0]non-4-ene-9-carboxylate was synthesized by cyclopropanation as described in literature.[5] After reduction with DIBAL-H to (1R,8S,9S,4Z)-Bicyclo[6.1.0]non-4-ene-9-ylmethanol photoisomerization led to cis-sTCO, following the protocol developed by Fox et al..[19] In a last step the PEG linker as well as the tosyl leaving group was introduced by reaction with triethylene glycol di(p-toluenesulfonate). This step proved to be problematic and even after optimization only a yield of about 30% could be achieved.

Tetrazine labelled RGD

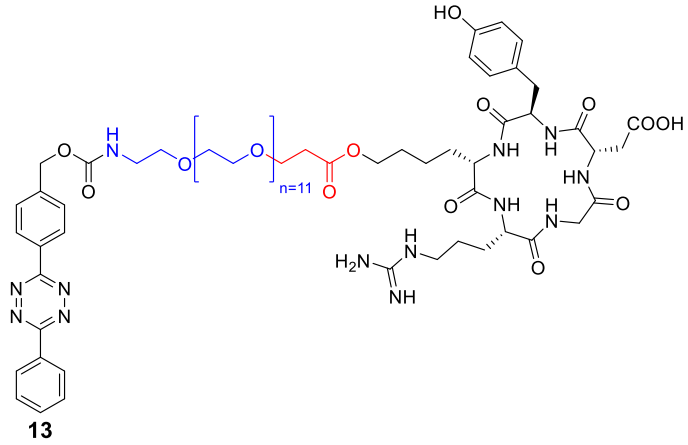
12 was synthesized by Dr. Yu Liu as shown in scheme 17. Unfortunately characterization of these compounds was difficult. Part of this project was the purification and characterization of compounds **10**, **11** and **12**.



Scheme 17: synthetic pathway to tetrazine labelled RGD **12**

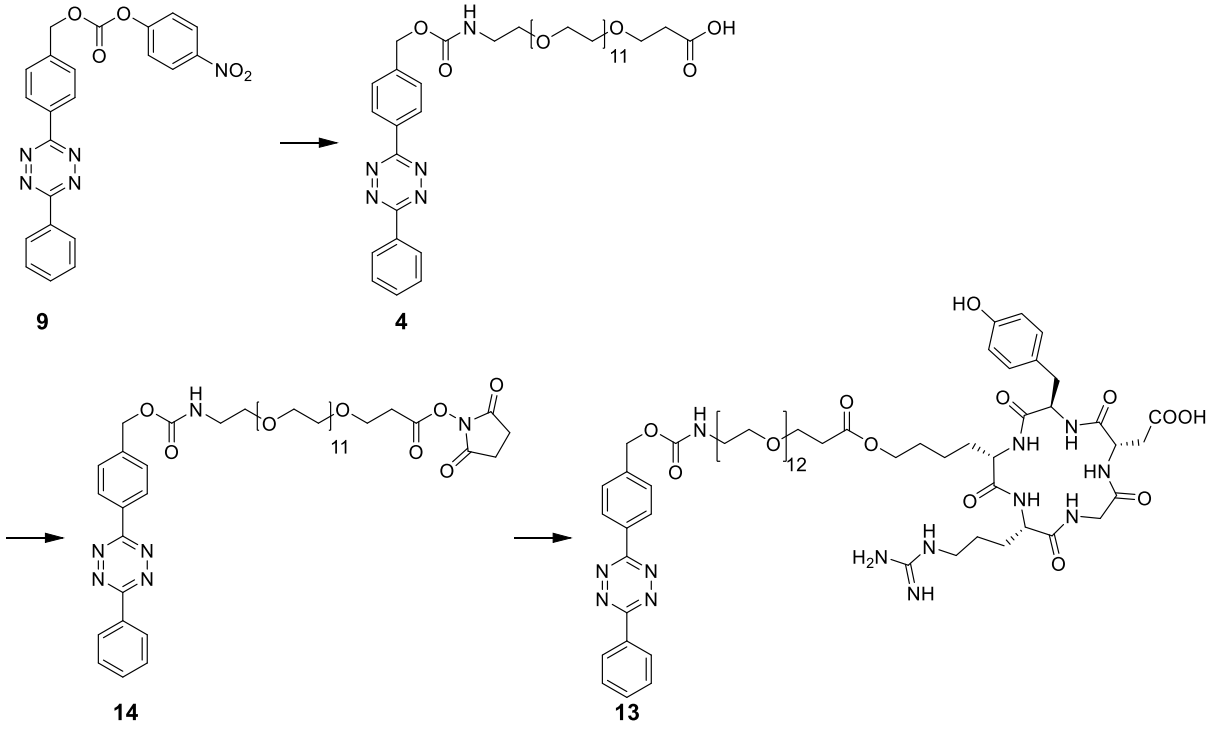
Therefore structure **10** and **11** was purified using preparative HPLC. Characterization of these compounds revealed, that the carboxylic acid group wasn't stable under acid conditions which were used for HPLC. This lead to re-synthesis of these compounds using slightly modificated purification steps to obtain pure substances. These pure compounds were submitted to HR-MS measurements using MALDI but no confirmation for this compounds could be obtained. We hypothesized that the compound absorbs the laser used in MALDI and gets destroyed while ionization.

This lead to redesign of the tetrazine moiety. Instead of a PEG 1000 spacer a shorter PEG spacer with a defined length of 12 PEG units was used (indicated in scheme 18 in blue). The acid group of the PEG chain was changed from the unstable 2-substituted acetic acid to a more stable 3-substituted propionic acid derivative (indicated in red).



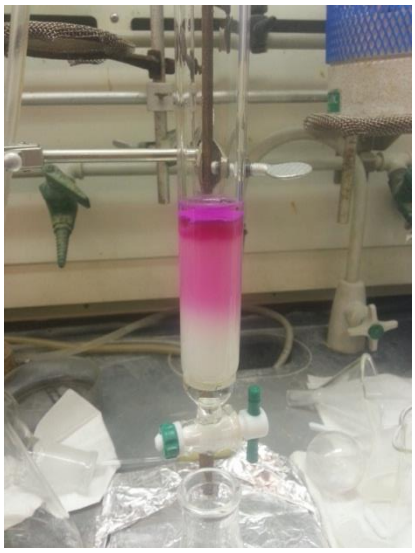
Scheme 18: redesigned tetrazine labelled RGD

Synthesis of this compound was done according to scheme 19.



Scheme 19: synthetic pathway to tetrazine labelled RGD **13**

Starting from 4-nitrophenolcarbonate **9** **4** was synthesized. EDCI coupling with NHS lead to compound **14**. Purification of this compound was difficult but could be achieved by applying HILIC on a preparative scale using 2.5 g silica gel and a solvent system of 5% H_2O in MeOH (picture 2 shows the column during purification). Coupling with cyclic RGD peptide and purification by preparative RP-HPLC lead to desired compound **13**.



Picture 2: HILIC column during purification of compound **14**

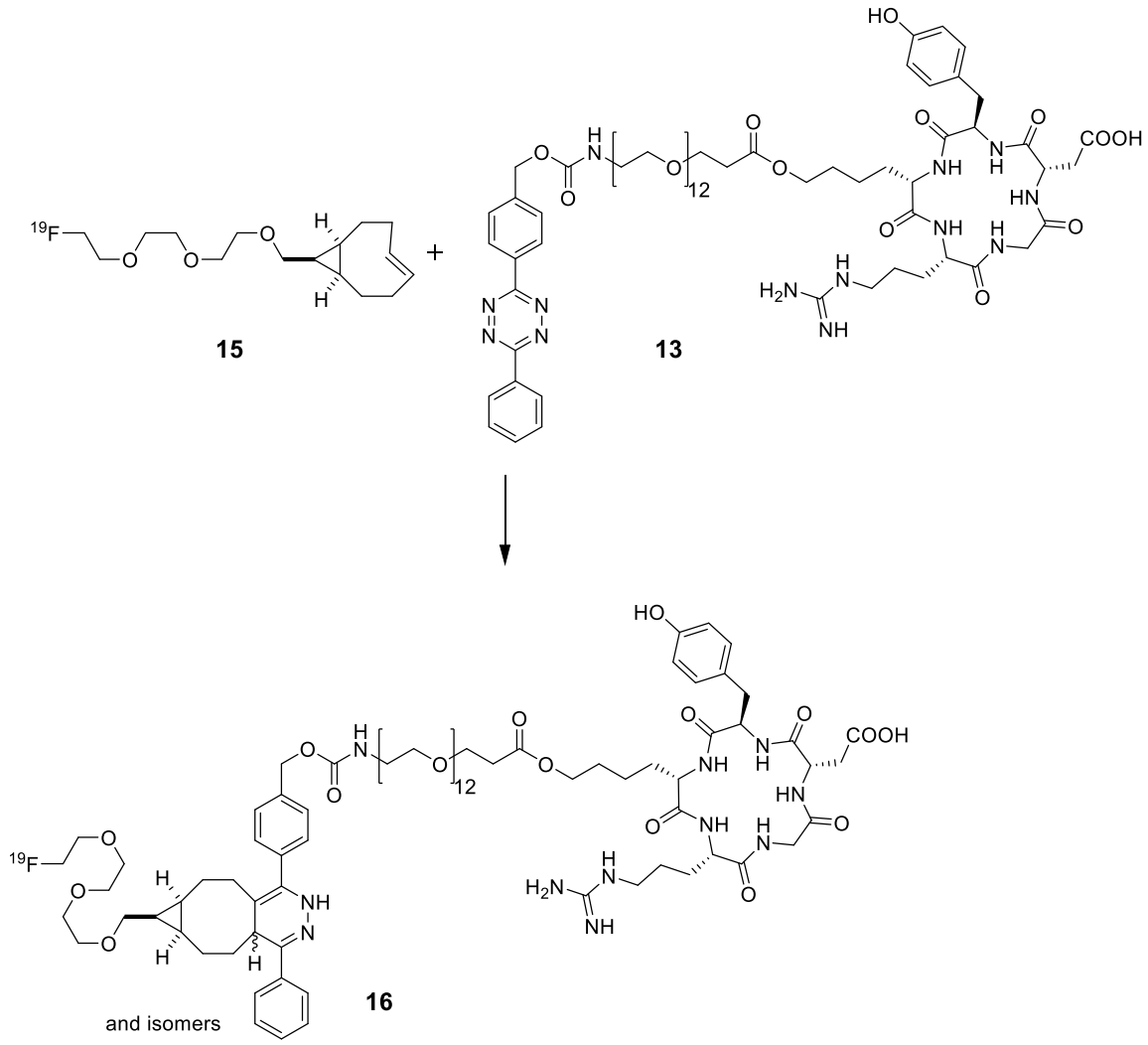
Synthesis of Reference Substances

Since radiolabelled substances are produced in very low quantities the only suited analytical methods are based on chromatography, mainly HPLC. Therefore "cold" (^{19}F labelled) reference substances are needed to confirm the outcome of radiosynthesis.

1 **15**

Scheme 20: Synthesis of cold reference **15**

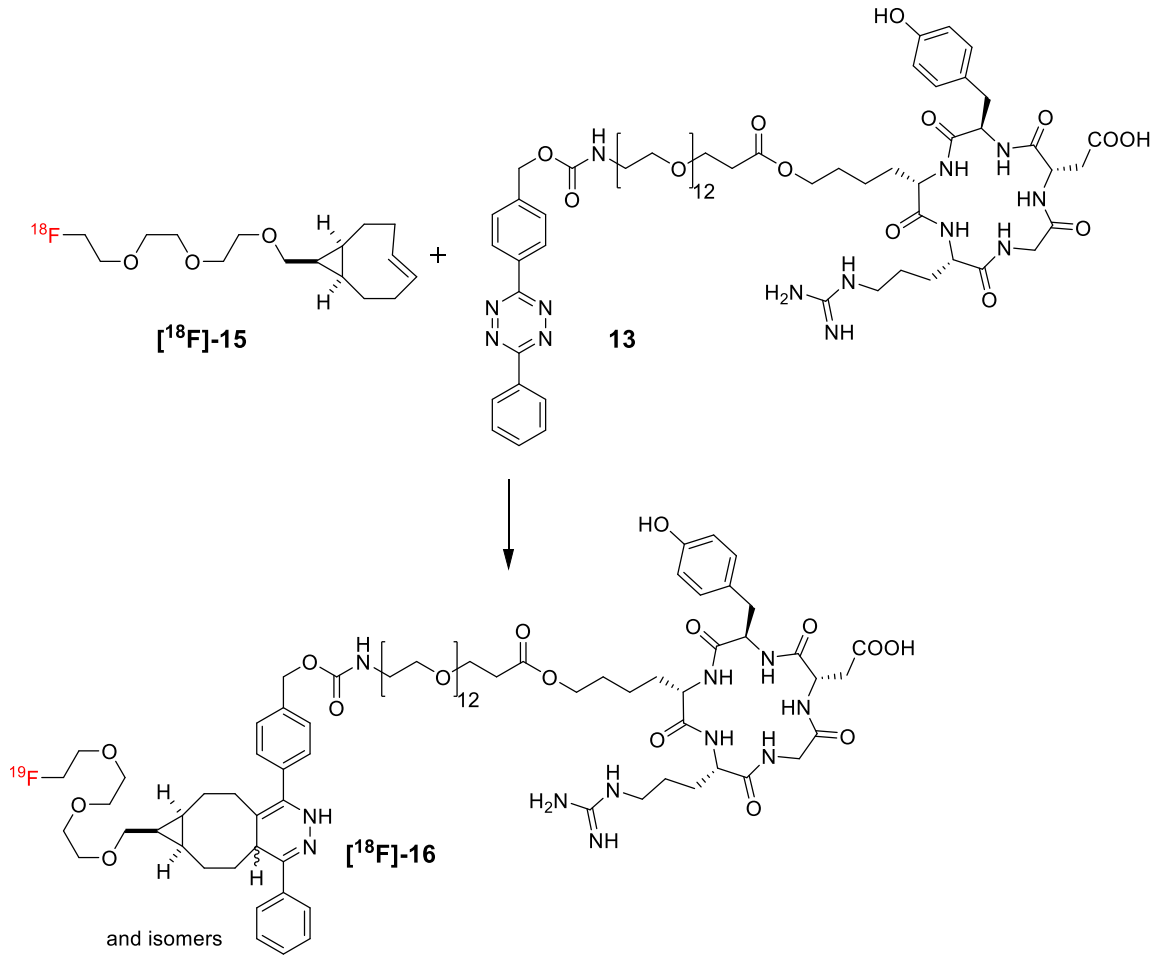
A cold (^{19}F substituted) reference of the radiolabelled sTCO was synthesized by heating **1** in a 1M solution of TBAF in THF (scheme 20). This compound was used to produce a cold reference of the radiolabelled RGD-peptide system according to reaction shown in scheme 21. This reaction was carried out on a 0.3 mg scale and the product was purified by prep-HPLC and identified using HR-MS.



Scheme 21: Synthesis of ligation product as reference material

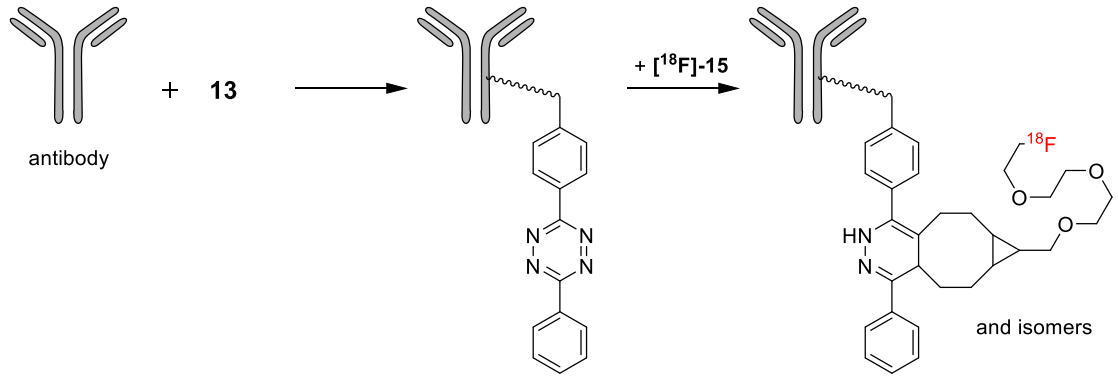
Further work.

The precursor **1** will be used for radiolabelling of sTCO giving compound **[^{18}F]-15**. Radiolabelled RGD [^{18}F]-**16** will be produced by rapid radiolabelling of **[^{18}F]-15** with **13** and used for imaging of U87MG tumor in mice, similar to work previously done by the groups of Fox and Li.[6–9] Radiosynthesis as well as animal studies are performed at University of North Carolina at Chapel Hill in the Group of Prof. Zibo Li.



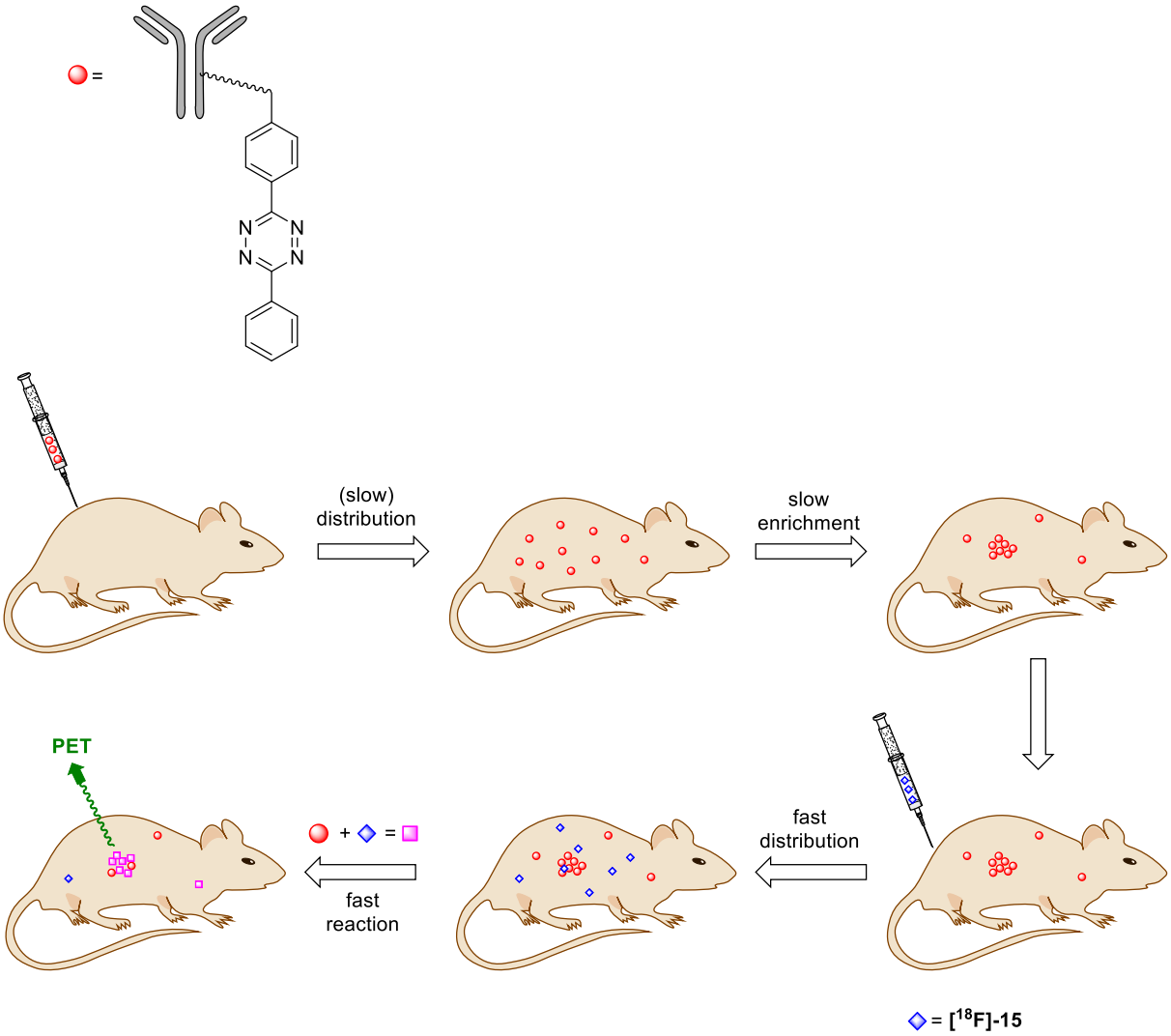
Scheme 22: synthetic pathway to radiolabelled compound **[^{18}F]-16**

Furthermore NHS ester **14** will be tested as tool for radiolabelling of antibodies, which could be modified with this compound by NHS-chemistry with tetrazines and rapid radiolabelling could be applied. (Scheme 23)



Scheme 23: potential rapid radiolabeling of antibodies using the technique described in this work

The last step would be the evaluation of this system for pretargeted PET imaging. Tetrazine labelled antibodies could be applied to mice followed by radiolabelled sTCO **[^{18}F]-15**. (Scheme 24)



Scheme 24: pretargeting approach using the system described in this work

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