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Bioorthogonal Imaging

Development of a ^{18}F-Labeled Tetrazine with Favorable Pharmacokinetics for Bioorthogonal PET Imaging**

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Abstract: *A low-molecular-weight ^{18}F-labeled tetrazine derivative was developed as a highly versatile tool for bioorthogonal PET imaging. Prosthetic groups and undesired carrying of ^{18}F through additional steps were evaded by direct ^{18}F-fluorination of an appropriate tetrazine precursor. Reaction kinetics of the cycloaddition with trans-cyclooctenes were investigated by applying quantum chemical calculations and stopped-flow measurements in human plasma; the results indicated that the labeled tetrazine is suitable as a bioorthogonal probe for the imaging of dienophile-tagged (bio)molecules. In vitro and in vivo investigations revealed high stability and PET/MRI in mice showed fast homogeneous biodistribution of the ^{18}F-labeled tetrazine that also passes the blood–brain barrier. An in vivo click experiment confirmed the bioorthogonal behavior of this novel tetrazine probe. Due to favorable chemical and pharmacokinetic properties this bioorthogonal agent should find application in bioimaging and biomedical research.*

Bioorthogonal imaging applying in vivo chemistry has emerged as a highly versatile tool in chemical biology and biomedicine.[1] Since the development of copper-free click chemistry for bioimaging using the strain-promoted azide–alkyne cycloaddition (SPAAC),[2] this bioorthogonal ligation has been used for a wide variety of applications with the aim to examine the molecular details of biological processes.[3] Although reaction kinetics were successively improved during the last years,[4] rate constants of SPAAC ligations are still limited ($< 5\,M^{-1}s^{-1}$, acetonitrile, 20 °C) and therefore this chemistry is not fully suitable for bioconjugations in vivo. Nevertheless, in vivo SPAAC has recently been applied for the development of a pretargeted imaging approach using positron emission tomography (PET).[5] To circumvent problems in terms of (relatively) low reaction rates of SPAAC ligations, Fox et al. and Weissleder et al. have independently introduced the inverse-electron-demand Diels–Alder (IEDDA)-initiated conjugation between 1,2,4,5-tetrazines (Tz) and strained cycloalkenes as a highly efficient bioorthogonal ligation.[6] Since then, this reaction has attracted broad attention and has been extensively used in many biomedical applications including the development of bioorthogonal probes, live-cell imaging, protein labeling, and the synthesis of PET imaging agents.[7] Furthermore, IEDDA was successfully applied in combination with SPAAC for simultaneous multitarget imaging.[8] Recently, Carlson et al. reported the development of advanced bioorthogonal turn-on probes that apply through-bond energy transfer leading to fluorescence quenching within boron dipyrromethene (BODIPY)-tetrazine derivatives.[9] Reaction kinetics of IEDDA ligations were further improved by the development and application of the bicyclo[6.1.0]non-4-ene (s-TCO) moiety, an even more strained *trans*-cyclooctene derivative, as a highly reactive dienophile leading to tetrazine ligations with rate constants of up to approximately $20000\,M^{-1}s^{-1}$ (MeOH, 25 °C).[10] In addition to commonly used *trans*-cyclooctenes (TCO) and s-TCO,[11] cyclopropene (CP) tags have recently been reported as useful dienophiles with lower steric demand and have successfully been applied in live-cell imaging.[12]

Despite many applications of IEDDA ligations in the field of fluorescence imaging, the development of bioorthogonal PET approaches is still restricted due to a lack of efficient radiolabeled agents that distribute homogenously and show fast in vivo reaction kinetics, high stability, and adequate clearance. ^{18}F-Labeling of dienophiles such as TCO and norbornene was preferred in terms of pretargeting approaches applying IEDDA bioconjugations so far due to the previously observed and reported insufficient stability of tetrazines during direct fluorination.[13] Yet, this approach is still cumbersome due to the fact that instead of readily available cycloalkene tags the tetrazine moiety has to be attached to the pretargeting probe. Furthermore, a tetrazine-type radiolabeled agent would be capable of being applied for bioorthogonal imaging of various dienophiles and thus provide high versatility. A ^{11}C-labeled tetrazine was initially developed by Herth et al.[14] and metal radionuclides such as ^{64}Cu and ^{89}Zr were attached to tetrazines using chelating ligands resulting in relatively large and complex bioorthogonally imaging agents.[15] Furthermore, polymer-modified tetrazines were developed and used for pretargeted PET imaging.[16] However, the development of a readily accessible radiolabeled low-molecular-weight tetrazine taking advantage of the high specific radioactivity of ^{18}F (allowing admin-

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istration of low-mass dosages and shorter radiation exposures) is still significant, which inspired us to focus on this particular problem in the field of bioorthogonal PET imaging.

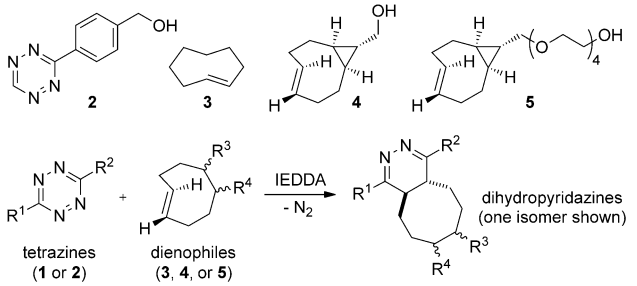
Herein we report our results on the synthesis and characterization of a low-molecular-weight ^{18}F-labeled tetrazine with favorable pharmacokinetics that is applicable as a versatile tool for pretargeted PET imaging using in vivo IEDDA chemistry.

During the development of improved procedures for the synthesis of 1,2,4,5-tetrazines, the characterization of a variety of these compounds revealed an inverse correlation between their stability in fetal bovine serum and the reaction rates of their cycloaddition with dienophiles.[17] Therefore, we hypothesized that stability problems during direct tetrazine fluorination can be avoided or minimized by using less reactive 3,6-dialkyltetrazines that still possess sufficiently high reaction rates for pretargeted in vivo applications. The availability of physiologically stable radiolabeled tetrazines is crucial for the development of reliable PET imaging approaches. In addition to this trade-off (reactivity vs. stability), we intended to develop water-soluble low-molecular-weight compounds with the aim to obtain imaging agents with favorable pharmacokinetic properties. These considerations led to the chemical structure of tetrazine **1**. The two alkyl substituents were chosen to be as small as reasonable. A derivative with a methyl group was preferred since it is more stable than an unsubstituted tetrazine, and a three-methylene spacer was considered between the fluorine and the tetrazine moiety to avoid the undesired high reactivity of benzyl-type precursors (Tz-CH_2-X) as well as possible elimination during nucleophilic fluorination (Tz-CH_2CH_2-X → Tz-CH=CH_2). Quantum chemical calculations on the basis of a recently reported computational approach (DFT, M06-2X/6-31G(d,p), Gaussian 09)[18] were applied to estimate the reaction kinetics (Table 1) of **1** in comparison to that of phenyl-substituted tetrazines (e.g. **2**),[19] which are commonly used in combination with TCO tags for various bioorthogonal ligation applications.[20] As expected, modeling of IEDDA reactions with *trans*-cyclooctene (**3**) showed an increased activation free energy for **1** (18.8 kcal mol^{-1}) relative to that for **2** (15.0 kcal mol^{-1}), but this difference is compensated when s-TCO **4** is used for cycloaddition with **1** (15.3 kcal mol^{-1}). Thus, applying the bioorthogonal reaction pair **1**/**4** should result in reasonable rate constants similar to those of the frequently applied ligation between **2** and **3**. This assumption was verified by stopped-flow measurements after preparation of **1** starting from 4-hydroxypropanenitrile (**6**) applying metal-catalyzed tetrazine synthesis[21] followed by DAST-mediated fluorination of the intermediate **7** (Scheme 1). Furthermore, a significantly increased reaction rate was observed for the cycloaddition of **1** with the water-soluble s-TCO derivative **5** in human plasma ($k_2 > 8000\ M^{-1}s^{-1}$, 37 °C), which is in agreement with previously reported studies on the effect of water on IEDDA reaction kinetics (Table 1).[22]

Regarding the preparation of compound **7**, we have studied the impact of different reaction parameters on tetrazine synthesis and have mainly focused on the reagent grade of hydrazine. Finally, we did not observe significantly decreased yields of **7** when the monohydrate (18 %) was used instead of anhydrous hydrazine (21 %) that was originally used for the metal-catalyzed preparation of 3,6-dialkyltetrazines.[21] This is of particular interest, since the availability of anhydrous hydrazine has been restricted in Europe since 2011 due to the REACH regulation of the European Chemicals Agency.[23] Although commercially available hydrazine cyanurate can be used as a reagent for the safe preparation of anhydrous hydrazine of high purity,[24] reliable synthetic methods using the monohydrate or other forms (e.g. hydrazine salts) should be preferred.

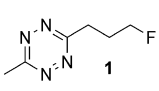
The in vitro stability of **1** in human plasma was assessed by spectrophotometric measurements and HPLC. Almost no degradation was observed after incubation at 37 °C for 12 h (99 % recovery) further revealing the capability of **1** as a promising scaffold for the development of a radiolabeled agent for pretargeted PET imaging. Although the excellent stability of **4** (no degradation in human serum, 30 mM butylamine in MeOH or 5 mM ethanethiol in MeOH) was shown by Taylor et al.,[10] they also observed isomerization (58 % after 3.5 h) to the unreactive *cis* isomer at a high concentration of ethanethiol (30 mM in MeOH). Furthermore, Rossin et al. have recently reported that *trans*-cyclooctenes are probably isomerized by interactions with copper-containing proteins; they also presented strategies to circumvent this problem by increasing the steric bulk of the tag.[25]

Table 1: Reaction kinetics of IEDDA cycloadditions obtained by computational methods (M06-2X/6-31G(d,p), Gaussian 09) and stopped-flow measurements.



Tetrazine	DP[a]	Solvent	$\Delta G^{\neq}_{calcd}$[b]	$\Delta G^{\neq}$[c]	k_2 [M^{-1} s^{-1}][d]
1	**3**	1,4-dioxane	18.7	17.5	1.49 ± 0.01
1	**4**	1,4-dioxane	15.3	15.0	85.5 ± 2.3
1	**5**	human plasma	n.d.[e]	n.d.	8200 ± 300
2	**3**	1,4-dioxane	15.0	15.1	79.2 ± 1.3

[a] DP = dienophile. [b] Calculated activation free energies (kcal mol^{-1}; 25 °C). [c] Activation free energies determined by stopped-flow measurements (kcal mol^{-1}; 25 °C). [d] Second-order rate constants (measurements at 37 °C). [e] n.d. = not determined.



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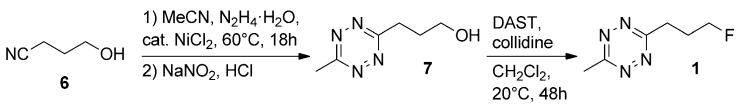

Scheme 1. Synthesis of tetrazine **1** (DAST = diethylaminosulfur trifluoride).

The stability of compound **5** was assessed by incubation in human plasma at 37 °C for 24 h leading to a recovery of > 80 %, a significantly higher value than those previously reported for reactive tetrazines.[17] Hence, the combination of radiolabeled tetrazines as pull-down reagents and (s-)TCO labeling should be advantageous for bioorthogonal in vivo imaging in contrast to inverse strategies involving tetrazine tags and radiolabeled dienophiles.

Radiosynthesis affording [^{18}F]-**1** was successfully carried out by direct fluorination of the tosylated intermediate **8** in a radiochemical yield of up to 18 % (Figure 1). Cycloaddition of [^{18}F]-**1** with s-TCO **4** occurred rapidly forming the conjugate [^{18}F]-**9** that slowly isomerized to [^{18}F]-**10** (both products as a mixture of regio- and stereoisomers).

In vivo studies were performed by administration of [^{18}F]-**1** to female BALB/c mice followed by dynamic PET ($n = 4$) and PET/MR ($n = 2$) imaging (MR = magnetic resonance tomography). A very homogenous biodistribution of [^{18}F]-**1** was observed in all regions in the body including the brain, which clearly indicates the ability of [^{18}F]-**1** to pass the blood–brain barrier (Figure 2). Furthermore, PET/MR revealed no significant extent of in vivo defluorination of [^{18}F]-**1**, since no enhanced radioactivity uptake in bone was observed.[26] Time–activity curves were similar for all analyzed organs

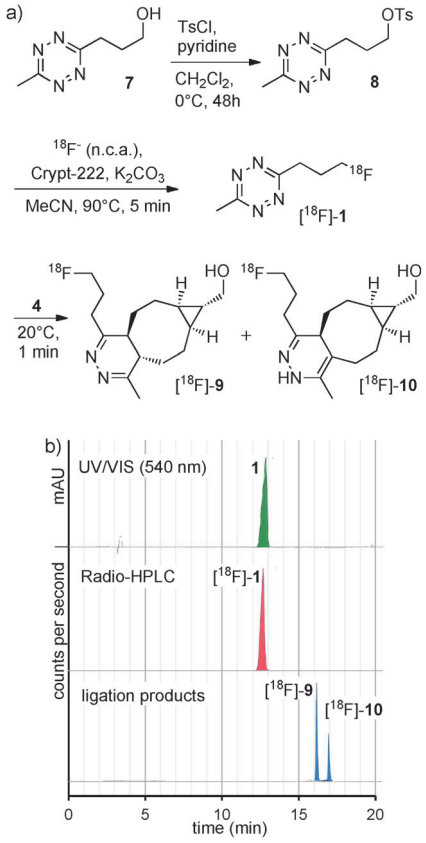

Figure 1. a) Radiosynthesis of [^{18}F]-**1** and IEDDA cycloaddition with s-TCO **4** (n.c.a. = no carrier added; only one isomer shown for [^{18}F]-**9** and [^{18}F]-**10**). b) HPLC of **1** (UV/Vis, 540 nm), radio-HPLC of purified [^{18}F]-**1** and ligation products after click reaction with s-TCO **4**. HPLC conditions: Zorbax SB-Aq reversed phase, H_2O/MeCN gradient elution.

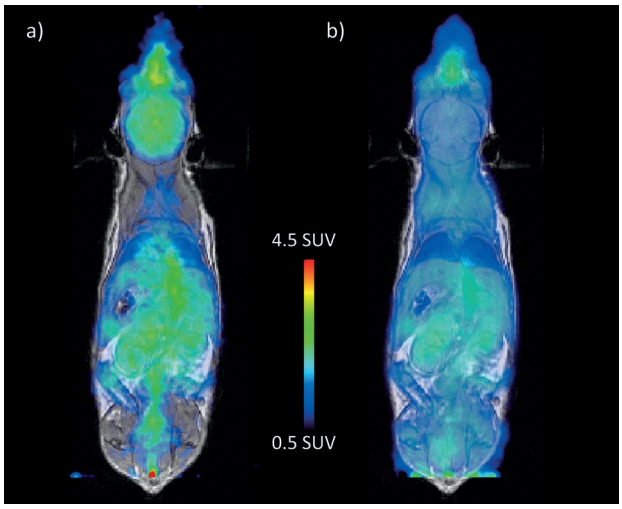

Figure 2. Combined PET/MRI summation images: 0–5 min (a) and 5–120 min (b) after injection of [^{18}F]-**1**.

(Figure 3 a). After the initial perfusion directly after intravenous administration of [^{18}F]-**1** a very uniform uptake steadily decreasing from 2 to 1.5 SUV (standardized uptake value) was observed in all organs (except the urinary bladder) during the onset of the PET scan. Conversely, SUV in the urinary bladder increased from 2 to 10 SUV, suggesting

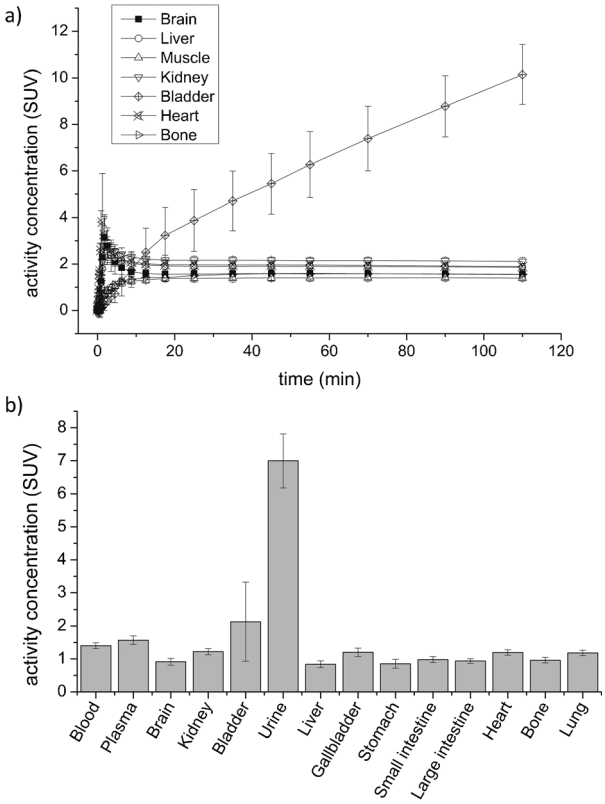

Figure 3. a) Mean organ time–activity curves obtained in BALB/c mice ($n = 6$; for bone $n = 2$). b) Ex vivo biodistribution of [^{18}F]-**1** in indicated organs 2 h after intravenous administration in mice ($n = 6$). Data are presented as mean SUV ± standard deviation. All organs (apart from the bladder) displayed similar radioactivity concentration values.

a rapid renal clearance of [^{18}F]-**1**. Evaluation of the ex vivo biodistribution determined 120 min after [^{18}F]-**1** injection gave values ranging from 1 SUV to 1.5 SUV with the exception of the urinary bladder (Figure 3b), which is in good agreement with the PET results. The in vivo stability of [^{18}F]-**1** was further examined by radio-TLC of mouse plasma obtained immediately after the imaging experiments (120 min after administration). We observed only a low degree of metabolic degradation (approximately 15%) as shown by the formation of more polar metabolites, which is assumed to occur via enzyme-catalyzed oxidation of the methyl group of [^{18}F]-**1** thus leading to xenobiotic metabolization similar to toluene and related compounds.[27]

Furthermore, the bioconjugation of [^{18}F]-**1** and the water-soluble dienophile **5** was performed in vivo to verify the bioorthogonal behavior of this novel tetrazine-type PET probe.[^{18}F]-**1** was administered to female BALB/c mice ($n = 4$) followed by **5** after an initial period of 20 min. In this experiment an inverse approach was used to allow for the homogenous biodistribution of the radiolabeled tetrazine prior to in vivo reaction. After 5, 15, and 30 min retroorbital blood was collected and the click reaction was quenched by addition of the more reactive tetrazine **2**. Murine plasma was obtained and analyzed using radio-TLC (Figure 4). Finally, we observed >90% conversion after 5 min and complete in vivo reaction 30 min after administration of **5** (see the Supporting Information, Figure S4). These results clearly indicate and confirm the capability of [^{18}F]-**1** as a highly valuable and versatile bioorthogonal probe for pretargeted PET imaging.

In summary, we have developed a fast and reliable procedure for the synthesis of a low-molecular-weight and water-soluble radiolabeled tetrazine; direct [^{18}F]-fluorination was applied to avoid additional steps and the need for prosthetic groups. Furthermore, [^{18}F]-**1** was prepared using readily accessible and/or commercially available reagents without the use of anhydrous hydrazine and [^{18}F]-**1** was shown to possess highly favorable pharmacokinetic properties. Although high stability of [^{18}F]-**1** was observed, its metabolic behavior has still to be verified in further studies. In vivo investigations clearly indicate the suitability of this radiolabeled tetrazine as a pull-down reagent for pretargeted PET imaging. Hence, we expect this highly versatile and readily accessible compound, which enables in vivo detection of dienophile-tagged (bio)molecules, to find many applications and lead to further investigations in the fields of bioorthogonal imaging and biomedical research.

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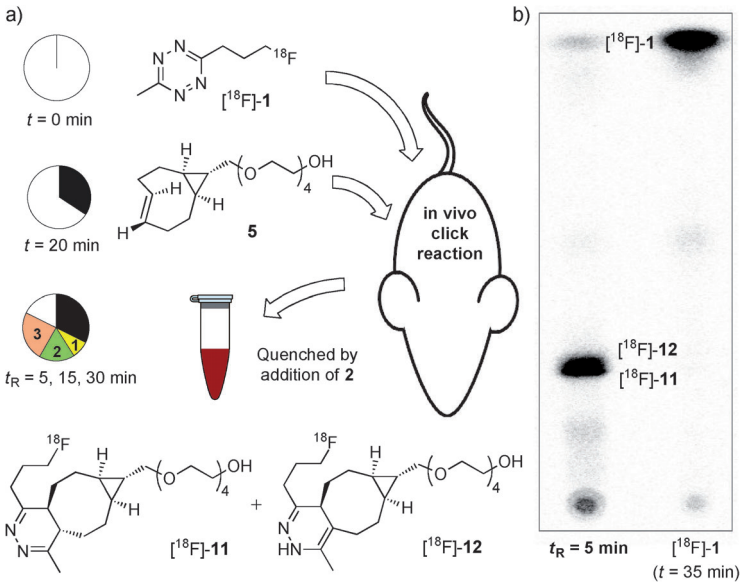

Figure 4. a) Bioorthogonal in vivo reaction of [^{18}F]-**1** and dienophile **5** leading to the formation of [^{18}F]-**11** and [^{18}F]-**12** (one isomer shown). b) Radio-TLC (SiO_2) of plasma obtained after 5 min of reaction time (left) and blank obtained 35 min after administration of [^{18}F]-**1** (right).

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