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Tetrazine-*trans*-Cyclooctene Ligation for the Rapid Construction of ^{18}F Labeled Probes

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Abstract

A radiolabeling method for bioconjugation based on the Diels-Alder reaction between 3,6-diaryl-*s*-tetrazines and an ^{18}F-labeled trans-cyclooctene is described. The reaction proceeds with exceptionally fast rates, making it an effective conjugation method within seconds at low micromolar concentrations.

Positron emission tomography (PET) is non-invasive imaging modality that utilizes positron-emitting radionuclides (C-11, N-13, O-15 and F-18).[1] F-18 PET has a number of attributes that make it clinically attractive, including 100% positron efficiency, a very high specific radioactivity, and a short half-life of ~110 min.[2] However, the short half-life of F-18 and the poor nucleophilicity of fluoride render it difficult to incorporate F-18 in complex molecules. Currently, radiochemistry is a major limiting factor for the field of PET.[3] Despite recent advances,[4,5] challenges exist for improving F-18 incorporation with respect to reaction rates, efficiency, and selectivity.

Recently, we introduced the tetrazine-*trans*-cyclooctene ligation ('TTCO ligation', Fig. 1) as a method of bioconjugation that proceeds with fast reaction rates without need for catalysis.[6,7] *trans*-Cyclooctene derivatives are readily prepared from *cis*-cyclooctenes using a photochemical flow-reaction that we developed.[8] While a variety of *s*-tetrazine derivatives were known react with strained alkenes,[9] we have found that 3,6-diaryl-*s*-tetrazines offer an excellent combination of fast reactivity and stability for both the conjugate and starting material.[6] In particular, 3,6-di(2-pyridyl)-*s*-tetrazines were shown to display excellent characteristics.[6,10] Thus, the reaction between *trans*-cyclooctene and **1a** proceeds with a rapid rate (k_2 ~ 2000 M^{-1} s^{-1} in 9:1 MeOH:water), and is successful in cell media and cell lysate.[6] 3,6-Di(2-pyridyl)-*s*-tetrazines can easily be functionalized as their amido derivatives (**1b**),[6] which display excellent stability toward water and biological nucleophiles.[10]

After we described the TTCO-ligation, the groups of Hilderbrand[11a] and Pipkorn and Braun[11b] described ligations between tetrazines and less reactive dienophiles. However,

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the use of *trans*-cyclooctene is the key to fast rates of reactivity, and other groups have subsequently developed applications for the TTCO-ligation.[11c-d,12]

Because of the fast rate of reactivity, the TTCO-ligation offers opportunities for the rapid conjugation of radionuclides to biomolecules, both of which are often available only at low concentration. Very recently, Robillard and coworkers elegantly demonstrated that our system for TTCO-ligation could be utilized in In^{111}–imaging of tumors in live mice using a DOTA-derivative of **1b**.[12] Herein, we report the development of an extremely fast and reactive method for generating ^{18}F labeled probes based on the TTCO-ligation.

While a number of indirect methods for ^{18}F-incorporation into tetrazines or *trans*-cyclooctene derivatives could be envisioned (e.g. derivatization by *N*-succinimidyl-4-^{18}F-fluorobenzoate[13]), such approaches are complicated by the need to carry ^{18}F through added manipulations. Thus, we focused on the development of direct methods for ^{18}F-incorporation via reactions with fluoride ion, with an initial focus on the synthesis of ^{18}F-labeled tetrazines (Scheme 1). Our attempts to convert nitrotetrazine derivatives **2a** and **2b** into ^{18}F-labeled substitution products **3** using ^{18}F-fluoride/kryptofix or ^{18}F-TBAF gave decomposition products and only traces of radiolabeled products. We also combined mesylate **4** with fluoride: in the most successful experiment (^{18}F-TBAF at 85 °C for 15 min), ^{18}F-labeled product **5** was obtained in ~1% labeling yield.

The instability of tetrazine precursors to F-labeling conditions prompted us to consider methods for preparing ^{18}F-labeled *trans*-cyclooctenes. To this end, we synthesized the nosylate **8** as shown in Scheme 2. The key step in the synthesis was photoisomerization of **6** to **7** using a flow reactor that continuously removes the *trans*-isomers through selective metal complexation.[7] The major diastereomer of **7** was carried forward in the synthesis of **8**.

Nosylate **8** reacted efficiently with TBAF to provide **^{19}F-9** in high yield. With this standard in hand, conditions for the preparation of **^{18}F-9** were optimized (Table 1). It was found that **^{18}F-9** could be obtained in good yield by adding nosylate **8** to a mixture of [^{18}F]-fluoride (100 mCi)/ tetrabutylammonium bicarbonate (TBAB) in acetonitrile (0.8 mL). We determined the effect of varying the concentration of **8** in reactions conducted for 15 min at 75 °C (Table 1, entries 1–4). The highest labeling yield (71%) was achieved with 7.0 mM **8**, but a useful radiochemical yield (18%) was still obtained with 0.35 mM **8**. Different reaction temperatures were studied, and 75 °C was found to be optimal (Table 1, entries 3, 5–7). Finally, we investigated the efficiency of the ^{18}F labeling as a function of time using 2 mg of **8** at 75 °C (Table 1, entries 8–10). While the labeling yield was optimal (71%) in an experiment conducted for 15 min, useful labeling yields were also obtained after 3 min (43%) and 7 min (68%). The conclusion of the experiments summarized in Table 1 is that the conditions of entry 3 (7.0 mM **8**, 75 °C, 15 min) are optimal for ^{18}F labeling.

As derivatives of 3,6-di(2-pyridyl)-*s*-tetrazine (e.g. **1b**, Figure 1) are readily accessible.[6,10,12] 3,6-di(2-pyridyl)-*s*-tetrazine (**1a**) was used to test the efficiency of the ^{18}F-labeled *trans*-cyclooctene **9** in the TTCO-ligation. A 'cold' study with **^{19}F-9** was initially conducted. As expected, the conjugate **^{19}F-10** formed immediately, and then slowly isomerized to 1,4-dihydropyrazine **^{19}F-11** as a mixture of isomers[6] (Scheme 3a). These isotopically stable conjugates served as coinjection standards for analysis of reactions with **^{18}F-9**.

The conjugations between **1a** and **^{18}F-9** were carried out in 1:1 acetonitrile/water, and were analyzed within 10 seconds of mixing. Prior to the conjugation, **^{18}F-9** was purified by HPLC and easily separated from unreacted precursor **8**. When **^{18}F-9** (1 mCi, 2 μM) was combined with **1a** (concentrations of ≥21 μM), **^{18}F-9** was completely consumed with 10 s, and **^{18}F-10** had formed in 98% radiochemical yield, accompanied by **^{18}F-11** (1%) (Table 2, entries 1-2).

When the concentration of ^{18}**F-9** was decreased to 0.1 mCi (0.2 μM), the conjugate **^{18}F-10/11** was still formed in excellent radiochemical yield (98%, entry 3). Useful radiochemical yields could also be obtained with even lower concentrations of **1a** (entries 4-5). We also investigated the efficiency of the conjugation between **1a** (21 μM) and ^{18}**F-9** (1 mCi, 2 μM) in PBS buffer and serum media: ^{18} **F-10** is formed in quantitative yield within 10 seconds (entries 6-7).

The Diels-Alder conjugate **10** was found to be stable in water, and the benign isomerization to **11** was the only side reaction. Thus, ^{19}**F-10** was the only product detected by ^{1}H NMR analysis immediately after the conjugation. In CD_3CN/H_2O, the rearrangement of ^{19}**F-10** to ^{19}**F-11** proceeded to 11% conversion after 4 hours, and >95% conversion after 48 hours. The stability of the radiolabeled conjugation product was monitored in PBS buffer and serum media for 4 hours, and no degradation products of ^{18}**F-10** were observed.

In conclusion, a new radiolabeling method for bioconjugation based on the TTCO-ligation has been described. The method utilizes a novel F-18 labeled *trans*-cyclooctene probe, and the efficiency of the reaction has been demonstrated with 3,6-di(2-pyridyl)-*s*-tetrazine, which can be readily functionalized via amido derivatives. The reaction proceeds with exceptionally fast rates, making it an effective conjugation method at low concentration. We anticipate that the TTCO-ligation will provide the foundation for a new class of reliable methods for ^{18}F-labeling of biomolecules in PET applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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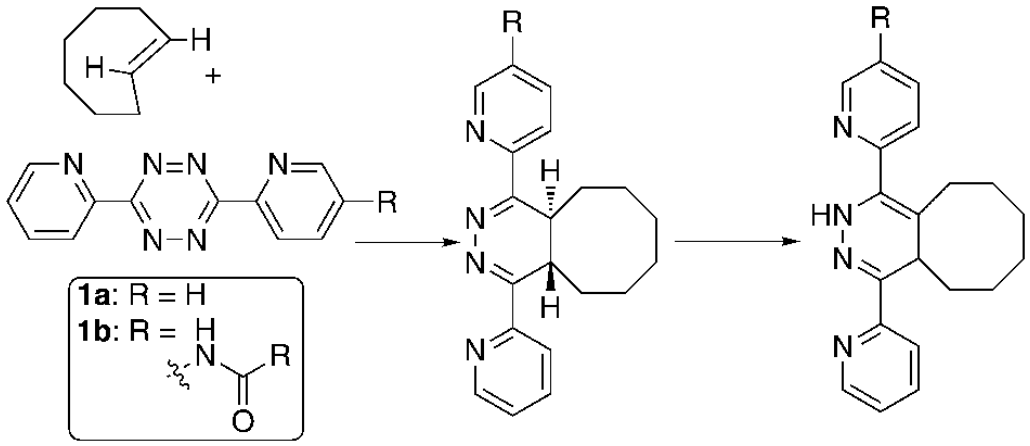
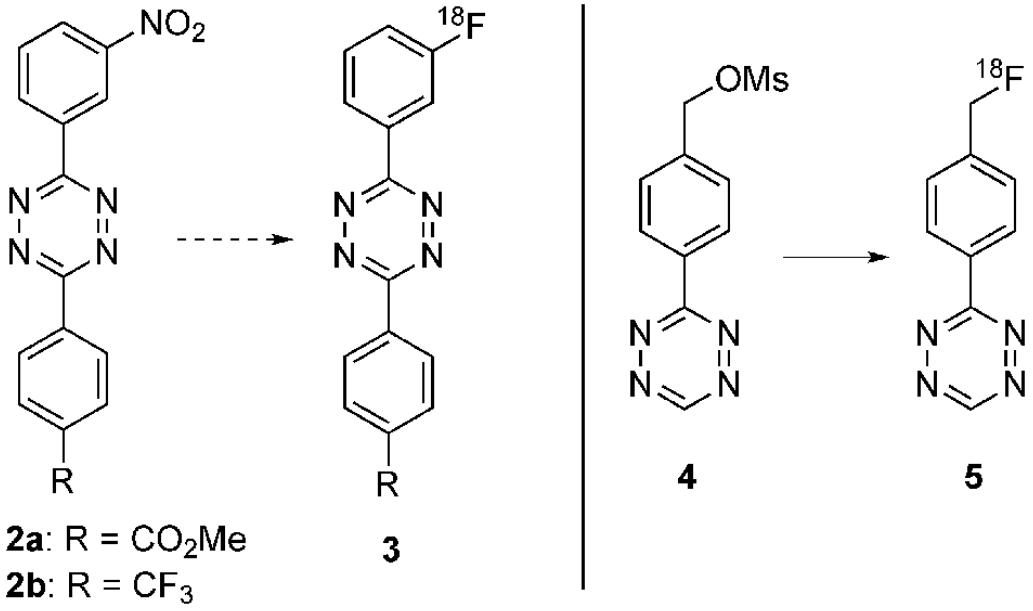
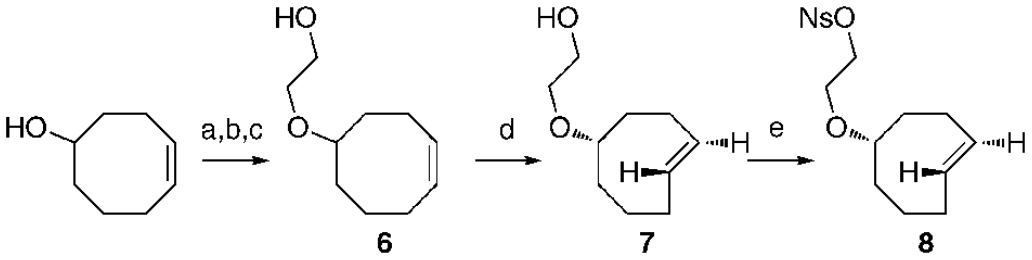


Fig. 1.
Tetrazine-*trans*-cyclooctene ligation ('TTCO-ligation')



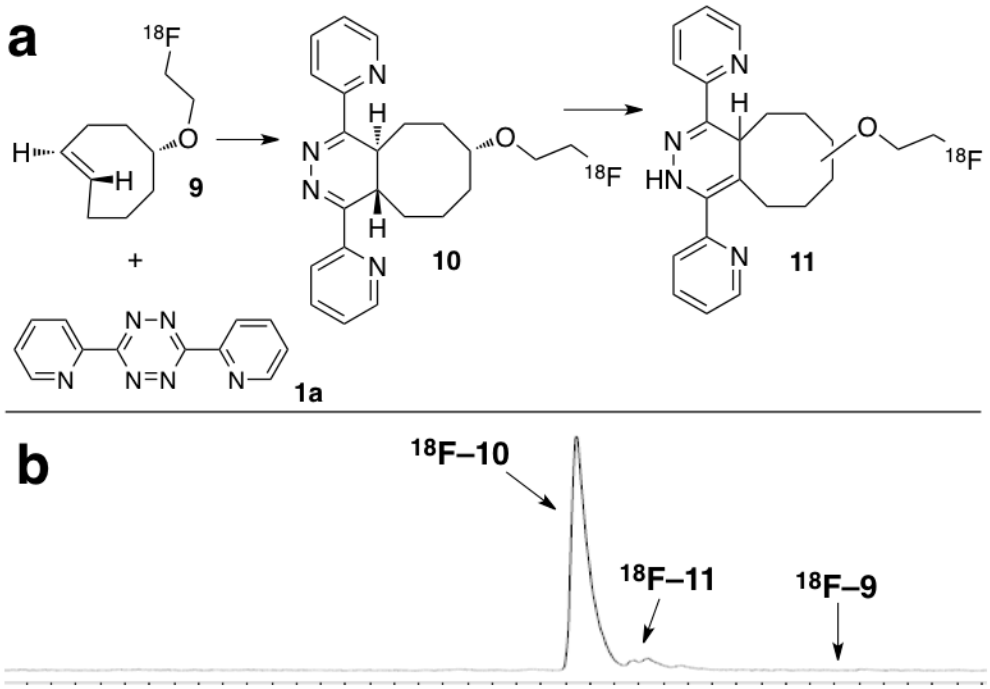
Scheme 1.

Synthesis of ^{18}F-labeled tetrazine. Attempts to prepare **3** by nucleophilic substitution were low yielding. Fluorination of **4** (^{18}F-fluoride, MeCN, 85 °C, 15 min) gives **5** in only 1% radiochemical yield.



Scheme 2.

Synthesis of *trans*-cyclooctene nosylate **8**. Reagents and conditions: (a) NaH, α-bromoacetic acid. (b) CH_2N_2, 69%, 2 steps (c) DIBAL, 78% (d) Methyl benzoate-sensitized photoisomerization with active removal of *trans*-isomers, $AgNO_3$, 55%. (e) NsCl, Et_3N, 87%.

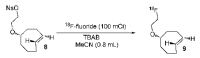


Scheme 3.

(a) Conjugation between **9** and **1a** occurs rapidly to form conjugate **10**, which slowly rearranges to **11** as a mixture of regio- and stereoisomers. (b) Radio-HPLC analysis of the crude product from Table 2 Entry 3

Table 1

Optimization of synthesis of ^{18}F-labeled *trans*-cyclooctene



Entry	Amount of 8	Temp	Reaction Time	Radiochemical Yield
1	100 μg (0.35 mM)	75 °C	15 min	18%
2	500 μg (1.8 mM)	75 °C	15 min	25%
3	**2.0 mg (7.0 mM)**	**75 °C**	**15 min**	**71%**
4	3.0 mg (11 mM)	75 °C	15 min	71%
5	2.0 mg (7.0 mM)	40 °C	15 min	24%
6	2.0 mg (7.0 mM)	55 °C	15 min	34%
7[a]	2.0 mg (7.0 mM)	90 °C	15 min	71%
8	2.0 mg (7.0 mM)	75 °C	3 min	43%
9	2.0 mg (7.0 mM)	75 °C	7 min	68%
10	2.0 mg (7.0 mM)	75 °C	30 min	71%

[a] Comparable results (70% RCY) were obtained under similar conditions using K_2CO_3/K_{222} instead of TBAB.

Table 2

The effect of concentration on the formation of **^{18}F-10** through the conjugation between **^{18}F-9** and 3,6-di(2-pyridyl)-*s*-tetrazine. All reactions were performed at room temperature.

Entry	^{18}F-9[a]	1a	Solvent	Reaction time	Radiochemical Yield (^{18}F-10 + ^{18}F-11)
1	1 mCi (2 μM)	210 μM	MeCN/H_2O	<10 s	>98 %
2	1 mCi (2 μM)	21 μM	MeCN/H_2O	<10 s	>98 %
3	0.1 mCi (0.2 μM)	21 μM	MeCN/H_2O	<10 s	98 %
4	0.1 mCi (0.2 μM)	2.1 μM	MeCN/H_2O	<10 s	56 %
5	0.1 mCi (0.2 μM)	0.21 μM	MeCN/H_2O	<10 s	15 %
6	1 mCi (2 μM)	21 μM	PBS buffer	<10 s	>98 %
7	1 mCi (2 μM)	21 μM	Serum	<10 s	>98 %

[a]The concentration of **^{18}F-9** was estimated based on the specific activity of fluoride after bombardment (~4 Ci/μmol), taking into account a correction for the rate of radioactive decay.