trans-Cyclooctene — a stable, voracious dienophile for bioorthogonal labeling

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Abstract

Discussed herein is the development and advancement of *trans*-cyclooctene as a tool for facilitating bioorthogonal labeling through reactions with *s*-tetrazines. While a number of strained alkenes have been shown to combine with tetrazines for applications in bioorthogonal labeling, *trans*-cyclooctene enables fastest reactivity at low concentration with rate constants in excess of $k_2 = 10^6$ M^{-1} s^{-1}. In the present article, we describe advances in computation and synthesis that have enabled applications in chemical biology and nuclear medicine.

Introduction

The use of strain energy to facilitate molecular reactivity is a key concept for organic synthesis, and one of emerging importance in the design of bioorthogonal reactivity — those chemical transformations that can proceed efficiently without interference from biological functionality [1,2•,3–14]. In a seminal publication in 2004, Bertozzi illustrated that the [3+2] cycloaddition between cyclooctyne and azide derivatives proceeds without the need for Cu-catalysis [15••]. As the scope and applications of this reaction have proven robust, so too has been the approach of using 'ring strain to promote otherwise reticent reactions with potential bioorthogonality' [15••]. Indeed, cycloalkynes have served as highly reactive dipolarophiles in a host of new bioorthogonal reactions [8,11,16–18].

Rate is a key consideration for the development of a bioorthogonal reaction, illustrated with the simple relationship between second order rate constant and half-life at 1 µM (Scheme 1b). A goal of this opinion article is to outline the development and advancement of *trans*-cyclooctene (TCO) as a tool for facilitating bioorthogonal labeling reactions with *s*-tetrazines. This multidisciplinary effort has embodied advances by a number of research groups in computation, synthesis, chemical biology and nuclear medicine (Scheme 1a).

Tetrazine–TCO ligation

Diels–Alder reactions between tetrazines and alkenes have a rich history [19], exemplified by classic physical organic studies of Sauer [20,21••,22] and the striking synthetic work of Boger [19,23]. In 1990, Sauer measured rate constants for the reactions of tetrazines with more than 40 dienophiles, including norbornene, cyclopropene derivatives, cyclooctyne and TCO [21••]. Of these dienophiles, TCO was most reactive by 1–3 orders of magnitude.

Our experience with the chemistry of strained alkenes [24,25•] led us to consider developing TCO for bioorthogonal reactivity with tetrazines [26•]. That the reaction of TCO with

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tetrazines would be bioorthogonal was not immediately evident, given the electrophilicity of tetrazine derivatives and the potential for TCO to isomerize to *cis*-cyclooctene or polymerize. Moreover, there was not a general synthetic method to access *functionalized* derivatives of TCO derivatives. Finally, while the reaction of TCO with *s*-tetrazines was known to be rapid, the conjugation product was undescribed and would warrant deconvolution.

As a general method to prepare TCO derivatives, we developed a photoisomerization method that uses a closed-loop flow reactor where the reaction mixture is continuously cycled through $AgNO_3$ on silica [25[•]]. Selective metal complexation of TCO perturbs an otherwise unfavorable equilibrium. This photochemical flow method can be carried out on multi-gram scale, and is robust enough to be useful in natural product synthesis [27].

With a TCO synthesis in hand, the bioorthogonality of tetrazine–TCO ligation was explored. 3,6-Diaryl-*s*-tetrazines offer an excellent combination of fast reaction rates and stability for both the starting material and conjugation products [28[••]], and protein conjugation at 15 μM was completed more quickly than could be easily assayed by mass spectrometry. While the initial product of tetrazine–TCO ligation is the expected 4,5-dihydropyridazine, this adduct reacts with water. In ^{1}H NMR studies of reactions between TCOs and 3,6-di(2-pyridyl)-*s*-tetrazine, it was shown that 4,5-dihydropyridazines (**4**) add D_2O to give aminal intermediates (**5**), which subsequently eliminate to give the 1,4-dihydropyridazine **6** (Scheme 1c). With more electron rich tetrazines, such as 3,6-diphenyl-*s*-tetrazine, dihydropyridazine **6** aromatizes to give **6a** under ambient conditions.

Contemporaneous with the above work on TCO, Weissleder and Hilderbrand studied the bioorthogonal reactivity of mono-aryltetrazine derivatives (**15**, Scheme 2c) with norbornenes with a rate of k_2 1.9 M^{-1} s^{-1} at 20 °C in PBS [29[••]]. Using a fluorescently labeled tetrazine, these authors demonstrated that the surfaces of live cells could be labeled. Contemporary studies were also performed by Pipkorn et al., who combined a TMZ-conjugate of tetrazine **14** with a peptide conjugate of the Reppe Anhydride [30[•]]. Recently, Devaraj and Presher have used cyclopropenes as dienophiles for cell surface labeling [31,32], and Wittmann has recently shown that terminal alkenes are sufficiently reactive to enable labeling by tetrazines [33]. Cyclooctynes have recently been used for reactions with tetrazines by Wang [34], and also in mutually bioorthogonal reactions [35,36]. Overall, there remains a high level of interest in TCO for achieving fastest reactivity.

Tetrazines used frequently in bioconjugation with TCO are listed in Scheme 2c [28[••],30[•], 37[••],38], and recently Devaraj has described a catalytic method for tetrazine synthesis from alkylnitriles [39[•]]. The reactions of tetrazines with TCO benefit from a significant hydrophobic effect. Thus TCO combines with 3,6-di(2-pyridyl)-*s*-tetrazine with a rate of 1100 M^{-1} s^{-1} in MeOH, and 2000 M^{-1} s^{-1} in 9:1 MeOH:water [28[••]]. Derivatives of **13** have rates of 5235 M^{-1} s^{-1} at 25 °C in 45:55 water:-MeOH [37[••]], and 13,090 M^{-1} s^{-1} at 37° in PBS [43[••]] (Scheme 2c). Very recently, Robillard has shown that a **13** derivative combines with the axial diastereomer of 5-hydroxy-*trans*-cyclooctene with a rate of 273,000 M^{-1} s^{-1} –1 at 37 °C in PBS — approximately 10 times faster than equatorial diastereomer [61]. As has been noted [41[•], 42[••]], the electron donating amido substituent renders **13** more stable than the parent 3,6-di(2-pyridyl)tetrazine, and derivatives of **13** have been used in a number of applications [37[••],41[•],43[••],44,45]. Contrary to several remarks in the literature [29[••],38,46], the stability of **13** in biological milieu appears comparable to other reactive tetrazines used for bioconjugation [38]. Weissleder and co-workers have reported fast rates for the reaction of monoaryltetrazines (**15**), where k_2 is 6000 M^{-1} s^{-1} in water at 37 °C [47[••]]. Derivatives of this tetrazine are cell permeable and have been used in a number of applications [29[••],35,38,46,47[••],48[••],49,50,51,52[•],53[•]] including the conjugation of

nanoparticles and quantum dots to pretargeted cells [49,50], as well as applications in cell and radiochemical imaging discussed below.

Applications in nuclear medicine

The radiochemical labeling of macromolecules represents one of the most significant opportunities for bioorthogonal chemistry. In 2010, Robillard and coworkers demonstrated that tetrazine–TCO ligation could be applied to pretargeted SPECT/CT imaging of tumors in live mice (Scheme 2a). Pretargeting involves separate administration of tumor-targeting and radiolabeled molecules, with time allowed in-between for uptake by tumors and clearance of any unbound targeting molecule [43••]. The success of bioorthogonal chemistry in pretargeted applications is critically dependent on reaction rate. Excess probe is required for reactions with slow rates, which can adversely impact the signal-to-noise ratio if the probe does not clear quickly. As depicted in Scheme 2a, 5-hydroxy-*trans*-cyclooctene was conjugated to anti-TAG72 mAb CC49 *via* an oligoethyleneglycol linker (**7**), and administered to mice bearing colon cancer xenografts. One day later, these mice were injected with 3.4 equivalents of a DOTA-In-111/tetrazine conjugate **8**, and tumors were successfully imaged by SPECT/CT. Impressively, the adduct **9** was formed in 52–57% yield *in vivo*. Recently, Robillard has found that tagging an antibody with a short linker and using an axial TCO diastereomer can improve the tumor-to-non-tumor imaging ratios in pretargeted tumor-bearing mice [61]. Also recently, Lewis and co-workers demonstrated pretargeted imaging of colorectal cancer in mice in a system using TCO-modified antibody and a Cu^{64}-NOTA labeled tetrazine [62].

TCO has also proven to be a powerful tool for positron emission tomography (PET), which is challenged by the short half-lives of PET radionuclides (110 min for ^{18}F). A further challenge is the ideal of achieving labeling with equimolar stoichiometry, as unlabeled precursors are generally inseparable and can decrease signal through competitive inhibition.

With Li and Conti, we developed ^{18}F-labeled TCO **11** (Scheme 2b) [54••], which was used to prepare cyclic RGD and VEGF protein conjugates for cancer imaging [41•,55]. The cyclic RGD targets the integrin $\alpha_v\beta_3$, which is a target for cancer imaging as it is upregulated on the surface of tumor blood vessels. We recently described a tetrazine-conjugate (**10**) of exendin-4 a 4.8 kDa glucagon like peptide-1 receptor (GLP-1R) [56•]. With only 2 equivalents (4 μg) of **10,** labeling by 0.02 mCi/mL of **11** was obtained to provide PET agent **12** in >80% yield within minutes. As shown in Scheme 2b, a GLP-1R positive tumor was successfully imaged by **12**. The importance of efficient ^{18}F incorporation was emphasized by a blocking experiment, where coinjecting **12** with a 5-fold excess of cold exendin-4 resulted in a greatly reduced signal. Compound **12** could be used to image intraportally transplanted islet cells, and it is a promising probe for monitoring the number and viability of transplanted islet cells in the liver.

Weissleder has conjugated ^{18}F-**11** to the drug AZD2281 for *in vivo* imaging applications, and used a TCO-functionalized resin to facilitate purification [51,52•]. Recently, ^{18}F-**11** was used for pretargeted imaging in conjunction with a dextran polymer modified by both a near-IR dye and tetrazine **15** [53•]. The dextran polymer was selected to improve pharmacokinetics. Mice were simultaneously implanted with LS174T tumors as well as A431 tumors that lack expression of the A31 glycoprotein epitope. The mice were administered a TCO-tagged anti-A33 antibody, and in second step were administered the modified polymer. The LS174T tumors were visualized whereas the control tumor showed much lower uptake [53•].

's-TCO' — a (more) strained *trans*-cyclooctene

The most reactive tetrazines, such as **13**–**15**, are imperfect when prolonged *in vivo* stability is required [43••]. Less electron deficient tetrazines, such as 3,6-diphenyl-*s*-tetra-zine, are much more resilient but are also less reactive toward TCO. van Delft has elegantly described that the strained cycloalkyne, bicyclo[6.1.0]non-4-yn-9-ylmetha-nol, displays superb reaction kinetics in bioconjugation chemistry [16]. To develop more reactive TCOs, we considered the effect that ring conformation would have on TCO reactivity. Computation was used to design a strained *trans*-cyclooctene ('s-TCO') with a *cis*-ring fusion, in which the eight-membered ring is forced to adopt a highly strained 'half-chair' conformation.

As summarized in Scheme 3a, *ab initio* calculations predicted the lowest energy 'crown' conformation (**2a**) of *trans*-cyclooctene to be 5.6–5.9 kcal/mol lower in energy than the 'half chair' conformation (**2b**) [17] and it was recognized that *trans*-cyclooctenes **18** with *cis*-ring fusions would be forced to adopt strained conformations similar to that of **2b** (Scheme 3b) [17,42••]. Transition state calculations with 3,6-diphenyl-*s*-tetrazine predicted that s-TCO **18a** would react much faster than **2a** ($\Delta\Delta G^{\ddagger\ddagger}$= 3.34 kcal/mol at 25 °C). Experimentally, **18b** was compared to **2**, and found to react in MeOH at 25 °C with a rate of 3100 M^{-1} s^{-1} — 160 times faster than **2** (Scheme 3b). The experimental $\Delta\Delta G^{\ddagger}$ (3.0 kcal/mol) was in close correlation to the predicted value.

Of course, even faster rates are observed with more reactive tetrazines. Reactions of s-TCO with 3,6-di(2-pyridyl)-tetrazine proceed with $k_2 = 22,000$ M^{-1} s^{-1} in MeOH at 25 °C [42••]. s-TCO also enjoys a significant hydrophobic effect. In 45:55 water:MeOH at 25° C, s-TCO **20** combines with **21** at a rate too fast to measure by stopped-flow kinetics (>200,000 M^{-1} s^{-1}) [37••]. Recently, the rate of s-TCO **18b** and **14** was measured to be 380,000 M^{-1} s^{-1} (7:1 water:dioxane, 25 °C) [64]. The rate of an s-TCO derivative with **8** was measured to be 2,800,000 M^{-1} s^{-1} at 37 °C in PBS [61].

Cellular imaging

Weissleder and co-workers first demonstrated that tetrazine–TCO ligation could be used to selectively label the surface (*via* pretargeted TCO-antibody conjugates) or interior (*via* TCO-taxol conjugates) of living cells with tetrazine–fluorophore conjugates [47••,48••,63]. These authors also demonstrated that tetrazines can quench the fluorescence of pendant dyes. As fluorescence is reestablished for Diels–Alder conjugates, background fluorescence is greatly reduced [47••,48••,63]. Cellular labeling has also been achieved with (*E,E*)-1,5-Cyclooc-tadiene *via* azido-tagged glycans on cell-surfaces [44].

Recently, a number of *in vivo* methods for the selective labeling of proteins based on tetrazine ligation have been developed. With Ting and co-workers, TCO-containing lipoic acid analogs were synthesized and an *E. coli* lipoic acid ligase was evolved that site-specifically ligates one of these TCO derivatives onto proteins of interest. Subsequent reactivity with tetrazine–dye conjugates served for efficient fluorogenic cell-surface labeling, and for intracellular labeling of cytoskeletal proteins in live mammalian cells [57••].

It has been shown with Mehl that tetrazine-containing amino acid **23** can be genetically encoded into proteins in site-specific fashion, and subsequently be labeled by TCO derivatives (Scheme 4a). The electron donating 3-amino substituent of **23** makes this tetrazine stable enough for cellular growth conditions, but also decreases the rate of Diels–Alder reactions. Fortunately, protein labeling by s-TCO **18b** and its derivatives occurs at

rapid rates. Thus, with GFP derivative **24**, fluorogenic labeling by **18b** takes place within minutes to give conjugate **25** [58••].

There has also been significant activity in the genetic incorporation of TCO containing amino acids. Independent work by Chin with Deiters [40], Carroll [59], and Schultz with Lemke [60••] established that norbornene could be incorporated and used for cell labeling. Schultz and Lemke also prepared the amino acid **26**, and were able to incorporate it into proteins and observe labeling in fixed cells, with a rate of 35,000 $M^{-1} s^{-1}$ at 37 °C *in vitro* [60••]. Independently with Chin, the amino acid **26** was synthesized and site specifically incorporated into proteins with high efficiency [37••]. Fluorogenic labeling was demonstrated in *E. coli* and living mammalian cells. Also studied was incorporation of an amino acid derived from the van Delft cyclooctyne [37••]. For those amino acids that incorporated into proteins, reactivity was fastest by an order of magnitude with **26** with rates of 5235 and 17,248 $M^{-1} s^{-1}$ (25 °C, 45:55 water:MeOH) with tetrazine derivatives of structures **13** and **14**, respectively (Scheme 2b).

Conclusion and outlook

Enabled by advances in synthesis and computation, TCO has emerged as a tool for rapid labeling and imaging through bioorthogonal Diels–Alder reactions with tetrazines. Future challenges include developing the process chemistry of TCO synthesis, as needed to broaden access. Another challenge will be to engage the most stable tetrazines with fast rates across a broader spectrum of applications. While s-TCO has proven successful in labeling genetically encoded tetrazines, encoding the s-TCO amino acid **20** was less successful (it isomerized), and attempts to utilize s-TCO **30** as a substrate for lipoic acid ligase met with low incorporation (Scheme 4b). New TCO designs that optimize structure, stability and rate will be needed to address such limitations.

Acknowledgments

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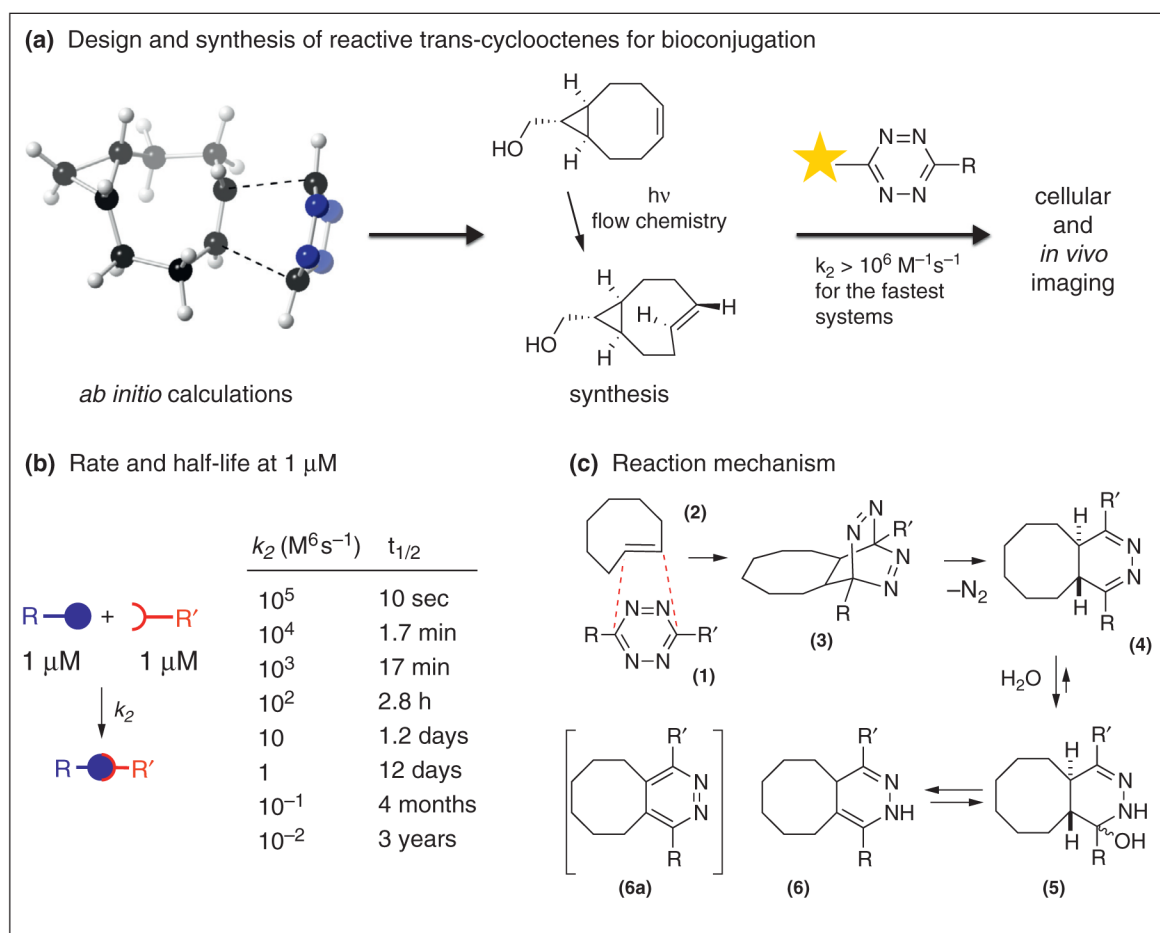
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(a) Design and synthesis of reactive trans-cyclooctenes for bioconjugation

ab initio calculations

synthesis

$h\nu$
flow chemistry

$k_2 > 10^6$ M^{-1}s^{-1}
for the fastest
systems

cellular
and
in vivo
imaging

(b) Rate and half-life at 1 μM

k_2 (M^{6}s^{-1})	$t_{1/2}$
10^5	10 sec
10^4	1.7 min
10^3	17 min
10^2	2.8 h
10	1.2 days
1	12 days
10^{-1}	4 months
10^{-2}	3 years

R—● +)—R'

1 μM   1 μM

k_2

R—●—R'

**(c)** Reaction mechanism

Scheme 1.
trans-Cyclooctene (TCO) as a tool for chemical biology and *in vivo* imaging. **(a)** The advancement of TCO as a tool is a multidisciplinary effort involving high-level computation, new synthetic methodology, chemical biology and nuclear medicine. **(b)** The significance of high rate constants is illustrated by simple table of rate data and corresponding half-lives. **(c)** Mechanism of the tetrazine–TCO ligation.
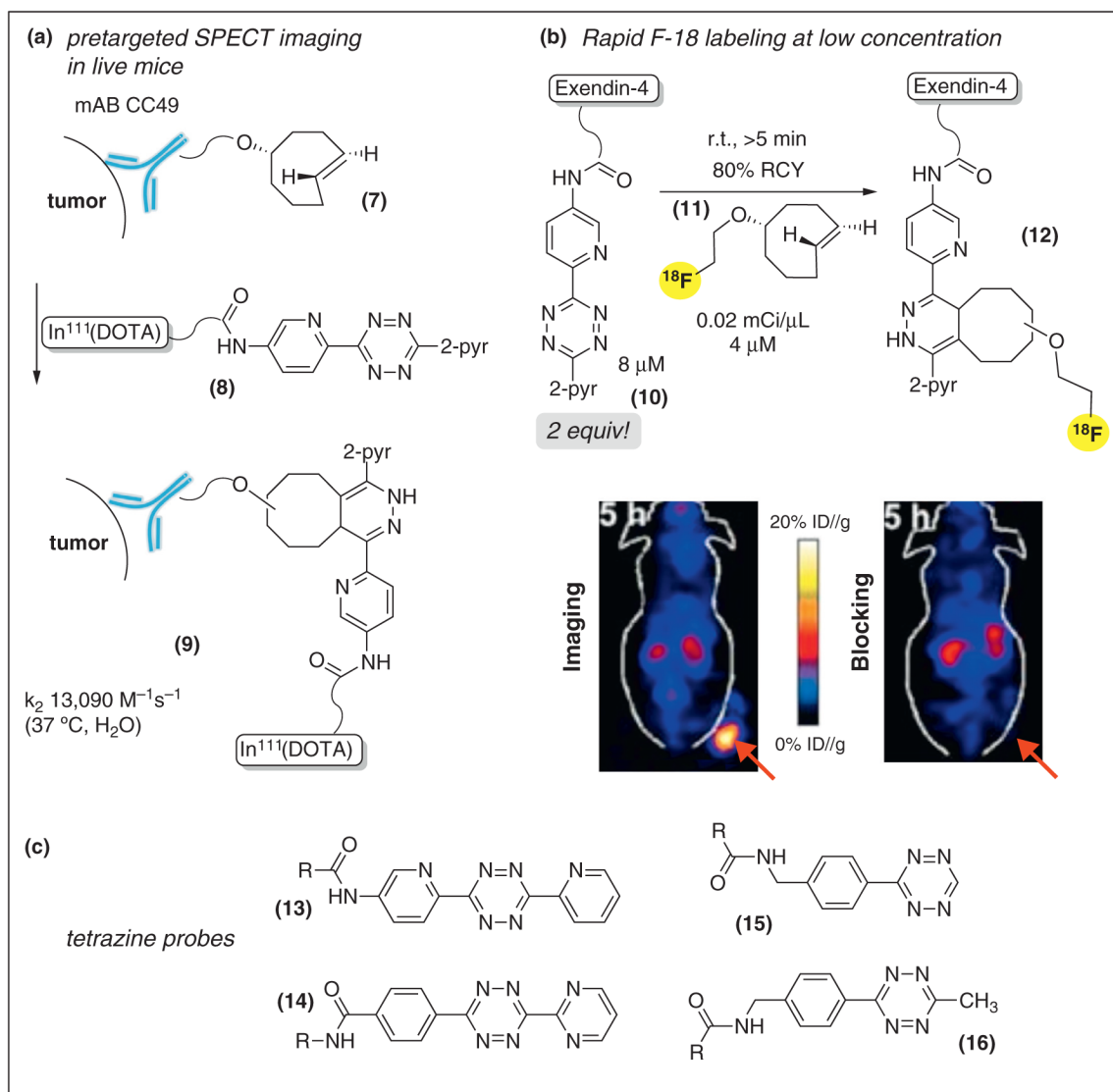
Scheme 2.
Tetrazine–TCO ligation as a tool for nuclear medicine **(a)** Pretargeted SPECT imaging in live mice through initial administration of a TCO-antibody conjugate with later administration of a tetrazine–[111]In-DOTA conjugate. **(b)** ^{18}F-labeled TCO **12** enables rapid construction of PET-probes. In the shown example, the importance of efficient labeling at nearly equimolar stoichiometry is emphasized by a blocking experiment, where coinjecting with a 5-fold excess of unlabeled exendin-4 resulted in a greatly reduced signal. **(c)** Tetrazines commonly utilized in bioconjugation studies.
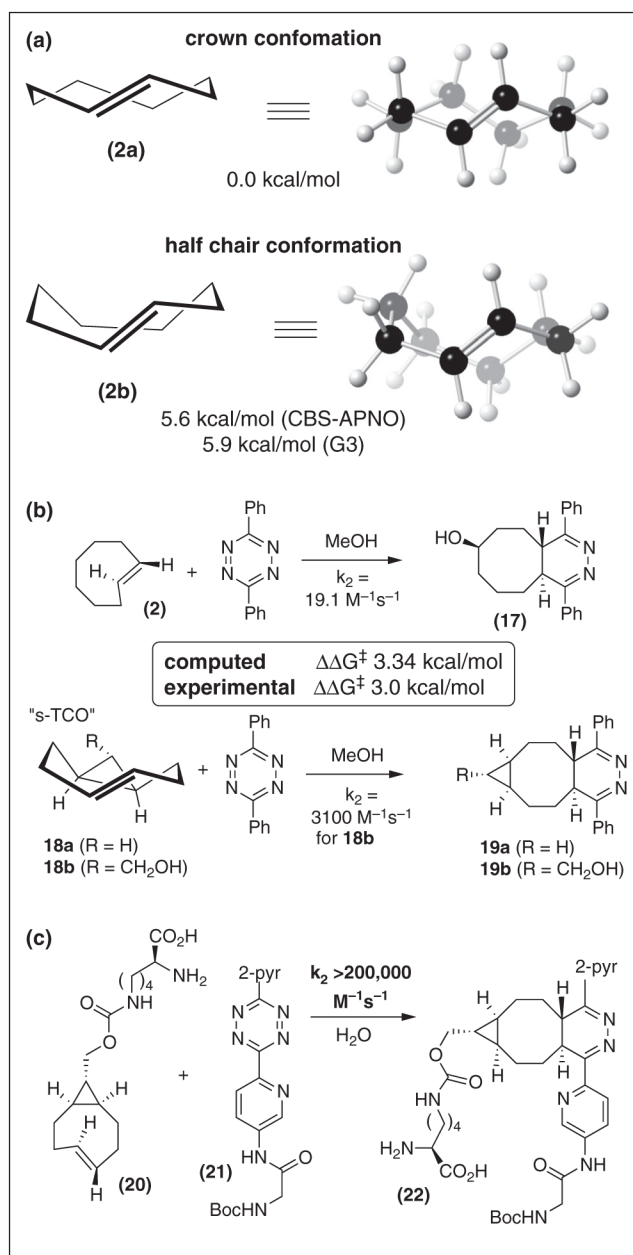
Scheme 3.
(a) TCO conformation has a significant effect on strain energy in the ground state. **(b)** Computation correctly predicted that a conformationally strained TCO ('s-TCO') would display enhanced reactivity relative to parent TCO. **(c)** With more reactive tetrazine **21**, s-TCO derivative reacts with a rate that is too quick to measure by stopped flow kinetics.
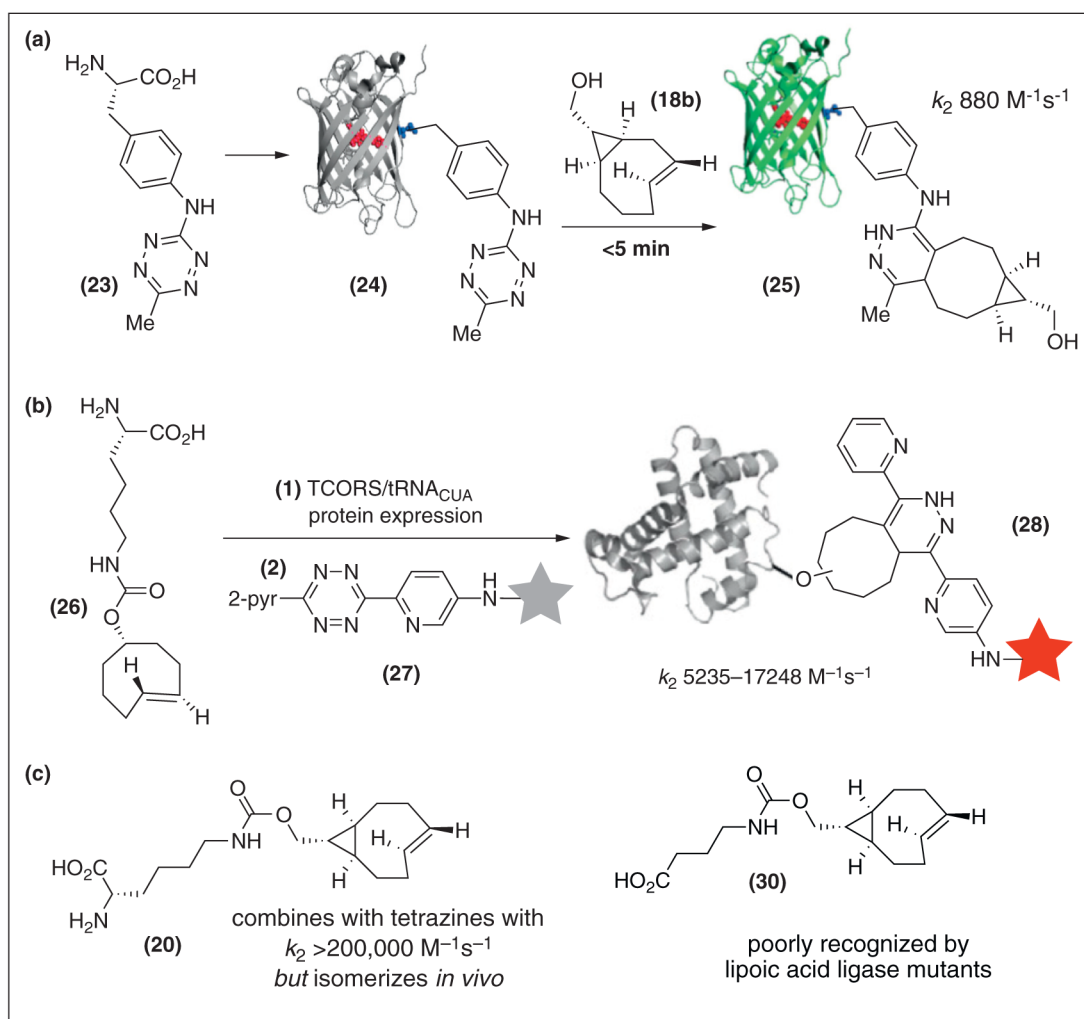
Scheme 4.
Tetrazine–TCO ligation with genetically encoded proteins **(a)** A tetrazine-derived unnatural amino acid can be genetically encoded site-specifically into proteins of interest. s-TCO derivatives can be used to tag the unnatural amino acids with fast rates *in vivo*. **(b)** A TCO-derivatized lysine has been site specifically incorporated into proteins in *E. coli* and mammalian cells, and used for rapid fluorogenic labeling in live cells. **(c)** Current limitations of s-TCO.