Uncovering the Key Role of Distortion in Bioorthogonal Tetrazine Tools That Defy the Reactivity/Stability Trade-Off

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ABSTRACT: The tetrazine/trans-cyclooctene ligation stands out from the bioorthogonal toolbox due to its exceptional reaction kinetics, enabling multiple molecular technologies in vitro and in living systems. Highly reactive 2-pyridyl-substituted tetrazines have become state of the art for time-critical processes and selective reactions at very low concentrations. It is widely accepted that the enhanced reactivity of these chemical tools is attributed to the electron-withdrawing effect of the heteroaryl substituent. In contrast, we show that the observed reaction rates are way too high to be explained on this basis. Computational investigation of this phenomenon revealed that distortion of the tetrazine caused by intramolecular repulsive N−N interaction plays a key role in accelerating the cycloaddition step. We show that the limited stability of tetrazines in biological media strongly correlates with the electron-withdrawing effect of the substituent, while intramolecular repulsion increases the reactivity without reducing the stability. These fundamental insights reveal thus far overlooked mechanistic aspects that govern the reactivity/stability trade-off for tetrazines in physiologically relevant environments, thereby providing a new strategy that may facilitate the rational design of these bioorthogonal tools.

INTRODUCTION

The inverse electron demand Diels−Alder (IEDDA)-initiated ligation of 1,2,4,5-tetrazines (Tz) with strained dienophiles represents a group of exceptionally fast bioorthogonal reactions.1−3 In particular, trans−cyclooctene (TCO) derivatives4−6 provide several orders of magnitude higher reactivity than other dienophiles such as cyclopropenes7 or norbornenes.8 In the rate-determining step, the tetrazine first reacts with the trans-cyclooctene in a [4 + 2]-cycloaddition reaction to form a bicyclic intermediate that rapidly undergoes cycloreversion to give dihydropyridazines as ligation products (Figure 1a). Due to high reaction rates, its biocompatibility, and versatility, the tetrazine/trans-cyclooctene ligation has found broad applications in many fields, in particular enabling selective chemical reactions in living organisms.2,5 Very recently, bioorthogonal chemistry has entered phase 1 clinical trials, with tetrazine/trans-cyclooctene reactions currently being tested in humans, aiming for locally restricted prodrug activation to improve the selectivity of chemotherapeutics.9,10

In recent years, a variety of differently substituted Tz have been used for bioorthogonal reactions and in vivo chemistry, including bis-alkyl-substituted Tz,11−15 alkyl-ary1-Tz,16−19 mono-alkyl-Tz20 (alkyl-H-Tz) as well as highly reactive bis-heteroary21,22 and mono-ary23−26 derivatives (aryl-H-Tz). These applications have motivated and fueled the development of advanced procedures for the synthesis of Tz scaffolds.27−31 In particular, 2-pyridyl-Tz are frequently used despite limited...
stability because of their exceptionally high reactivity. This is commonly attributed to the electron-withdrawing effect of the 2-pyridyl substituent, resulting in a lowered orbital energy of the Tz, thereby accelerating the IEDDA cycloaddition (Figure 1b).\(^{2,3,32}\) Assuming that the reactivity is indeed controlled by frontier molecular orbital (FMO) interactions, we hypothesized that 4-pyridyl-substituted Tz are even more reactive than their 2-pyridyl analogues.\(^{33-36}\) So far, 4-pyridyl-Tz (Figure 1b) have only rarely been used,\(^{36}\) and there is no comparative data on the IEDDA reaction kinetics of 2-pyridyl- and 4-pyridyl-Tz. In addition, vinyl ether-Tz, recently introduced by Fox et al.,\(^{29,30}\) have been shown to exhibit increased IEDDA reactivity despite the non-electron-withdrawing nature of the substituent (Figure 1b), while also maintaining high stability. Intrigued by these findings, we speculated about the existence of yet overlooked mechanistic aspects other than FMO interactions that have a crucial effect on the reactivity of Tz.

Here, we present the results of a combined experimental and computational approach, finally revealing the key role of Tz distortion in the bioorthogonal reaction with trans-cyclooctenes. We show that this effect accelerates both 2-pyridyl- and vinyl ether-substituted Tz, thereby providing fundamental insight into the underlying mechanism and a molecular basis to describe the reactivity and stability of these bioorthogonal tools.

## RESULTS AND DISCUSSION

First, we aimed to study the influence of aryl substituents on the click reactivity with TCO and therefore selected a series of phenyl (Ph), 2-pyridyl (2Pyr), 3-pyridyl (3Pyr), and 4-pyridyl (4Pyr)-substituted Tz (Figure 2a) for initial investigations. Theoretical calculations were performed using density functional theory (DFT) at the \(\omega\)B97X-D/6-311G(d,p)-SMD(1,4-dioxane) level of theory, and orbital energies were calculated at the HF/6-311+G(d,p)-SMD(1,4-dioxane)//\(\omega\)B97X-D/6-311G(d,p)-SMD(1,4-dioxane) level of theory. A detailed description of the computational methods used within this study can be found in the Supporting Information. Energies for the reacting LUMO+1 of Tz range from 1.29 to 0.89 eV. As expected, Ph shows the highest orbital energy, followed by 2Pyr and 3Pyr, which were calculated to have an almost equal LUMO+1 level, while 4Pyr shows the lowest orbital energy (Figure 2a). According to FMO theory, 4Pyr should thus indeed show an increased IEDDA reactivity compared to 2Pyr.

To compare these results with the measured reaction rates, we have prepared all selected mono-substituted aryl-Tz using a method recently published by Audebert and co-workers.\(^{27}\) The rate constants for the ligation with TCO were measured to be governed by FMO interactions, 2Pyr is significantly more reactive (>3-fold) than expected based on the respective orbital energy (Figure 2c). Qualitatively equivalent results were obtained using the calculated Kohn–Sham orbital energies (see Supporting Information, Figure S1). Hence, the high IEDDA reactivity of 2Pyr cannot be attributed to the electron-withdrawing effect of the 2-pyridyl substituent.

To investigate the origin of the observed reactivity trend for different pyridyl substituents, a DFT study of the respective reactions was performed, showing good correlation between the calculated free energies of activation (\(\Delta G^\ddagger\)) and the experimental values (Figure S2). The calculated transition-state geometries revealed a slight asynchronicity with very similar forming bond lengths across all four Tz. However, 2Pyr shows a slightly more synchronous bond formation (Figure 3a).

For a detailed investigation of the barrier heights, we performed a distortion/interaction analysis (also referred to as the activation/strain model)\(^{38}\) on all four transition states (Figure 3b) using the auto DIAS software package.\(^{39}\) This energy decomposition method was introduced independently by Houk and Bickelhaupt and has successfully been applied to investigate bioorthogonal cycloadditions.\(^{52,40-42}\) In this analysis, the energy of activation (\(\Delta E^\ddagger\)) is dissected into two parts, the distortion energy (\(\Delta E_{\text{dist}}\)), which is needed to distort the isolated reactants into transition-state geometry, and the interaction energy (\(\Delta E_{\text{int}}\)) resulting from bringing the two distorted isolated fragments together. The analysis was performed at the transition state as a defined point for each reaction. Performing the distortion/interaction analysis at the transition state geometry can lead to skewed results if transition states at highly different forming bond lengths are compared.\(^{35}\) However, here the forming bond lengths are very similar in all cases, warranting the comparison at the transition state. To validate these results, an analysis at a consistent geometry with fixed bond lengths for all Tz was conducted, which qualitatively provided the same results (see Supporting Information, Table S2). Interaction energies were calculated to be similar for all studied reactions (within 1.1 kcal mol\(^{-1}\)) and do not explain the observed differences in IEDDA reactivity. However, \(\Delta E_{\text{int}}\) for 2Pyr is about 1.5 kcal mol\(^{-1}\) lower than that for Ph, 3Pyr, and 4Pyr, demonstrating that the increased reactivity of 2Pyr with TCO is mainly caused by a reduced distortion energy (Figure 3b).

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**Figure 2.** (a) LUMO+1 orbitals and orbital energies of mono-substituted Tz showing a significantly lower LUMO+1 energy for 4Pyr; (b) rate constants for the reaction of Ph, 2Pyr, 3Pyr, and 4Pyr with TCO (1,4-dioxane, 25 °C, \(n = 6, \text{SD} < 1\%\)); (c) measured rate constants vs calculated LUMO+1 energy.
To explain the lowered $\Delta E_{\text{dist}}$ for aryl substituents with nitrogen atoms in 2-position, we have focused on the geometries encountered at the transition state. In all cases, the aryl moiety is tilted away from the allylic CH$_2$ of TCO. For Ph, 3Pyr, and 4Pyr, the dihedral angle is approximately 80°, which we reasoned is due to the steric demand of the allylic CH$_2$. This hypothesis is in agreement with the investigations of analogous reactions with ethylene (no allylic CH$_2$), showing dihedral angles of approx. 90° (Figures 4a and S3). However, for 2Pyr, we observed a much stronger tilt in the transition state for the reaction with TCO, with a dihedral angle of 63°, which did not change in the reaction with ethylene. These observations demonstrate that it is an intrinsic property of 2-pyridyl-substituted Tz rather than forced by steric interactions. In fact, the calculated geometry of 2Pyr revealed that a nitrogen–nitrogen interaction destabilizes the reactant. This interaction becomes apparent when looking at the stabilization energies when going from an orthogonal (i.e., dihedral angle between the two aromatic rings of 90°) conformer in the reactant to the stable, coplanar conformer (Figure 4b). For Ph, 3Pyr, and 4Pyr, this stabilization energy based on the conjugation between the ring systems is approximately 6 kcal mol$^{-1}$. However, for 2Pyr, the most stable conformation is not planar, and the stabilization is only 3.5 kcal mol$^{-1}$ due to a repulsive interaction between the pyridyl nitrogen and the vicinal Tz nitrogen. This repulsion counteracts the stabilization due to the conjugation of the aromatic systems, leading to an almost flat energy surface between $-30$ and $+30$°, with a minimum at a dihedral angle of 12° (Figure 4b). At the transition state, this N–N repulsion can be avoided by the rotation of the substituent, thereby increasing the distance between the interacting nitrogen atoms. The nitrogen lone pairs then point in different directions, further reducing the repulsive interaction. In addition, natural bond orbital analysis revealed (i) a weak intermolecular hydrogen bond between the pyridyl nitrogen of 2Pyr and the vinylic CH of TCO, similar to the interactions previously described for bioorthogonal 1,3-dipolar cycloadditions$^{44}$ and (ii) an increased $n_N \rightarrow \sigma^*_{C-N}$ donation of the pyridyl lone pair into the $\sigma^*$ of the Tz C–N bond.$^{45}$ However, both these weak interactions were found to play a minor or negligible role regarding the observed rotation in the transition state (see Supporting Information, Figure S4).

In summary, our results revealed that the high reactivity of 2-pyridyl-Tz cannot be explained by the electron-withdrawing nature of the heteroaryl substituent only but moreover by intramolecular N–N repulsion, finally uncovering the mechanistic key role of distortion in Tz ligations.

To confirm that these findings can be translated to physiological conditions, additional IEDDA reactions were carried out in Dulbecco’s phosphate buffered saline (DPBS) at 37 °C. Due to the limited stability of the mono-substituted H-Tz in aqueous solution, we have prepared the respective methyl-Tz MePh, Me2Pyr, Me3Pyr, and Me4Pyr and determined the second-order rate constants for the reaction with water-soluble TCO–PEG$_4$ (Figure 5a). This TCO
stability was monitored by spectrophotometry (Figure 5b).

Encouraged by the computational results, we have prepared the vinyl ether-Tz MeEVE and the 3,4-dihydro-2H-pyran (DHP)-substituted Tz MeDHP and DHP (Figure 7a; for details on synthetic procedures, see the Supporting Information), but did not obtain sufficient amounts of pure material of the respective bis(vinyl ether)Tz EVE₂ [3,6-bis(1-

predicted by FMO theory but also shows a significantly higher stability than expected based on its IEDDA reactivity. Based on these key mechanistic insights and considering the mentioned vinyl ether-substituted Tz developed by Fox and co-workers (Figure 1b), we hypothesized that the observed unexpectedly high reactivity of these Tz is due to the lowered distortion energies caused by intramolecular O–N repulsion (Figure 6a), analogous to N–N repulsion in 2-pyridyl-

To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco's modified Eagle's medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than MePh, Me2Pyr, Me3Pyr, and Me4Pyr in buffered aqueous solution (DPBS) at 37 °C using water-soluble TCO-PEG₄ (n = 6, SD < 1%), (b) stability of Tz in cell growth medium at 37 °C (n = 3) revealing accelerated degradation with decreasing LUMO+1 energy as a measure for the increased electron-withdrawing character of the (hetero)aryl substituent, promoting the attack of nucleophiles (Nu).

It is generally accepted that increasing the reactivity of Tz by using electron-withdrawing substituents leads to reduced stability in aqueous/biological media due to accelerated attack of nucleophiles causing Tz degradation (Figure 5b). However, when working with buffered aqueous solutions of the aryl-methyl-Tz, we noticed a significantly faster degradation of Me4Pyr compared to Me2Pyr (as indicated by accelerated fading of the characteristic pink color of Tz), despite the higher reactivity of the 2-pyridyl-Tz. To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco's modified Eagle's medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than MePh, Me2Pyr, Me3Pyr, and Me4Pyr compared to Me2Pyr (as indicated by accelerated fading of the characteristic pink color of Tz), despite the higher reactivity of the 2-pyridyl-Tz. To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco’s modified Eagle’s medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than MePh, Me2Pyr, Me3Pyr, and Me4Pyr compared to Me2Pyr (as indicated by accelerated fading of the characteristic pink color of Tz), despite the higher reactivity of the 2-pyridyl-Tz. To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco’s modified Eagle’s medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than MePh, Me2Pyr, Me3Pyr, and Me4Pyr compared to Me2Pyr (as indicated by accelerated fading of the characteristic pink color of Tz), despite the higher reactivity of the 2-pyridyl-Tz. To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco’s modified Eagle’s medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than MePh, Me2Pyr, Me3Pyr, and Me4Pyr compared to Me2Pyr (as indicated by accelerated fading of the characteristic pink color of Tz), despite the higher reactivity of the 2-pyridyl-Tz. To investigate this observation, MePh, Me2Pyr, Me3Pyr, and Me4Pyr were incubated in full cell growth medium (Dulbecco’s modified Eagle’s medium, DMEM, incl. 10% fetal bovine serum) at 37 °C, and Tz stability was monitored by spectrophotometry (Figure 5b). Indeed, Me4Pyr degraded much faster (30% intact after 12 h) compared to Me3Pyr and Me2Pyr (approx. 85%) and MePh (>95%). These results correlate well with the decreasing LUMO+1 energy of the Tz (Figure 5b), which we used as a measure for the increased electron-withdrawing effect of the (hetero)aryl substituent. This finding is in agreement with the hypothesis of accelerated nucleophilic attack leading to Tz degradation. Me2Pyr is thus not only more reactive than
ethoxyvinyl-Tz; structure not shown]. Second-order rate constants for the reactions of these Tz with TCO-PEG4 in DPBS at 37 °C were determined by stopped-flow spectrophotometry. The IEDDA reactivity of MeEVE (2750 M⁻¹ s⁻¹) was shown to match the value measured for Me4Pyr (2740 M⁻¹ s⁻¹). In comparison, the cyclic vinyl ether-Tz MeDHP was observed to be less reactive (1820 M⁻¹ s⁻¹), though still significantly faster than the aryl-Tz MePh (990 M⁻¹ s⁻¹). As expected, we observed a high reactivity of the bis-vinyl ether-Tz DHP₂ (6450 M⁻¹ s⁻¹), exceeding the rate constant of Me2Pyr (5120 M⁻¹ s⁻¹) by approx. 25% (Figure 7a). These results finally confirm the distortion-induced IEDDA acceleration due to intramolecular O–N repulsion and that increased reactivities similar to pyridyl-Tz can be achieved by using non-electron-withdrawing vinyl ether substituents.

Subsequent investigation of Tz stability in full cell growth medium at 37 °C moreover confirmed the high stability of vinyl ether-Tz MeEVE (in accordance with previous findings) and, in particular, MeDHP and DHP₂, in contrast to the limited stability of pyridyl-substituted Tz (Figure 7b).

For instance, despite being equal in reactivity, MeDHP is significantly more stable than 4Pyr (>90% vs <15%). Notably, DHP substituents do not lead to decreased stability, as shown by the data obtained for symmetrical DHP₂ and bis(2-pyridyl)Tz (2Pyr₂). While installation of a second DHP had no detrimental effect on stability (>90% for both MeDHP and DHP₂), an additional 2-pyridyl substituent resulted in almost complete Tz degradation within 24 h. Despite showing very fast IEDDA reaction with TCO-PEG₄ (69,400 M⁻¹ s⁻¹), only <1% of intact 2Pyr₂ was detected at the end of the experiment, in comparison to >90% of DHP₂ (Figure 7b). In applications that require an extended stability of Tz (>10 h), DHP₂ thus outperforms even highly reactive 2Pyr₂ (Figure S5). Overall, these results confirm that non-electron-withdrawing DHP substituents can be used to significantly increase the IEDDA reactivity of Tz while maintaining a high compound stability (Figure 7c).

■ CONCLUSIONS

Our detailed investigation of the reactions of pyridyl-Tz with TCOs uncovered the key role of reduced Tz distortion energies caused by repulsive intramolecular interactions. Based on these insights, we have been able to confirm an analogous effect in the case of vinyl ether-Tz and showed that 3,4-dihydro-2H-pyran (DHP) substitution increases IEDDA reactivity without accelerating Tz degradation under physiological conditions. Overall, we provide a new mechanistic understanding that may be instrumental in the rational design of next-generation bioorthogonal tools with enhanced reactivity and stability, particularly for strategies that require or benefit from long-term Tz stability.

■ ASSOCIATED CONTENT

+ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.2c01056. Computational methods, organic synthesis, reaction kinetics, compound stability and characterization, and copies of NMR spectra (PDF)
Coordinates of all calculated geometries (ZIP)

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Notes
The authors declare no competing financial interest.

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■ REFERENCES

(1) Nguyen, S. S.; Prescher, J. A. Developing bioorthogonal probes to span a spectrum of reactivities. Nat. Rev. Chem. 2020, 4, 476–489.
(2) Mayer, S.; Lang, K. Tetrazines in Inverse-Electron-Demand Diels–Alder Cycloadditions and Their Use in Biology. Synthesis 2017, 49, 830–848.
(3) Oliveira, B. L.; Guo, Z.; Bernardes, G. J. L. Inverse electron demand Diels-Alder reactions in chemical biology. Chem. Soc. Rev. 2017, 46, 4895–4950.
(4) Selvaraj, R.; Fox, J. M. trans-Cyclooctene—a stable, voracious dienophile for bioorthogonal labeling. Curr. Opin. Chem. Biol. 2013, 17, 753–760.
(5) Darko, A.; Wallace, S.; Dmitrenko, O.; Machovina, M. M.; Mehl, R. A.; Chin, J. W.; Fox, J. M. Conformationally Strained trans-Cyclooctene with Improved Stability and Excellent Reactivity in Tetrazine Ligation. Chem. Sci. 2014, 5, 3770–3776.
(6) Pinzger, J. E.; Rosenberger, J. E.; Jemsa, A.; Boyd, S. J.; Dmitrenko, O.; Xie, Y.; Fox, J. M. Angew. Chem., Int. Ed. 2021, 60, 14975–14980.
(7) Yang, J.; Seckute, J.; Cole, C. M.; Devaraj, N. K. Live-Cell Imaging of Cyclopropene Tags with Fluorogenic Tetrazine Cycloadditions. Angew. Chem., Int. Ed. 2012, 51, 7476–7479.
(8) Knall, A.-C.; Hollauf, M.; Sulgovc, C. Kinetic studies of inverse electron demand Diels–Alder reactions (iEDDA) of norbornenes and 3,6-dipyrindin-2-yl-1,2,4,5-tetrazine. TetraED酮 Lett. 2014, 55, 4763–4766.
(9) Wu, K.; Yee, N. A.; Srinivasan, S.; Mahmoodi, A.; Zakharian, M.; Mejia Oneto, J. M.; Rozen, M.; Krivak, P.; Doseck, U.; de Geus, M. A. R.; Maurits, E.; Doelman, W.; Overkleeft, H. S.; Codée, J. D. C.; Filipov, D. V.; van Kesteren, S. I. Fast and pH-Independent Elimination of trans-Cyclooctene by Using Aminoethyl-Functionalized Tetrazines. Chem.—Eur. J. 2018, 24, 18075–18081.
(10) Wilkovicth, M.; Haider, M.; Sohr, B.; Herrmann, B.; Klubnick, J.; Weissleder, R.; Carlson, J. C. T.; Mikula, H. A Cysymmetrical trans-Cyclooctene Enables Fast and Complete Bioorthogonal Disassembly of Molecular Probes. J. Am. Chem. Soc. 2020, 142, 19132–19141.
(11) Rossin, R.; Renart Verkerk, P.; van den Bosch, S. M.; Vuldur, R. C. M.; Verel, I.; Lub, J.; Robillard, M. S. In Vivo Chemistry for Pretargeted Tumor Imaging in Live Mice. Angew. Chem. Int. Ed. 2010, 49, 3375–3378.
(12) Störz, E. J. L.; Jørgensen, J. T.; Denk, C.; Battisti, U. M.; Noregngard, K.; Edem, P. E.; Brattely, K.; Shalgunov, V.; Wilkovicth, M.; Svatunek, D.; Poullie, C. M. B.; Hvass, L.; Simón, M.; Wane, T.; Rossin, R.; Robillard, M.; Kristensen, J. L.; Mikula, H.; Kjaer, A.; Herth, M. M. Lipophilicity and Click Reactivity Determine the Performance of Bioorthogonal Tetrazine Tools in Pretargeted In Vivo Chemistry. ACS Pharmacol. Transl. Sci. 2021, 4, 824–833.
(13) Meimatis, L. G.; Carlson, J. C. T.; Giedt, R. J.; Kohler, R. H.; Weissleder, R. Ultrafluorogenic Coumarin–Tetrazine Probes for Real-Time Biological Imaging. Angew. Chem., Int. Ed. 2014, 53, 7531–7534.
(14) Zeglis, B. M.; Sevak, K. K.; Reiner, T.; Mohindra, P.; Carlin, S. D.; Zanonzico, P.; Weissleder, R.; Lewis, J. S. A Pretargeted PET Imaging Strategy Based on Bioorthogonal Diels–Alder Click Chemistry. J. Nucl. Med. 2013, 54, 1389–1396.
(15) Denk, C.; Wilkovicth, M.; Anheim, E.; Herth, M. M.; Jensen, H.; Lindegren, S.; Mikula, H. Multifunctional Clickable Reagents for Rapid Bioorthogonal Stabilization and Radio-Crosslinking. ChemPlaChem 2019, 84, 775–778.
(16) Garcia-Vázquez, R.; Battisti, U. M.; Jørgensen, J. T.; Shalgunov, V.; Hvass, L.; Stares, D. L.; Petersen, I. N.; Crestey, F.; Löfler, A.; Svatunek, D.; Kristensen, J. L.; Mikula, H.; Kjaer, A.; Herth, M. M. Direct Cu-mediated aromatic 18F-labeling of highly reactive tetrazines for pretargeted bioorthogonal PET imaging. Chem. Sci. 2021, 12, 11668–11675.
(17) Qu, Y.; Sauvage, F.-X.; Clavier, G.; Miomandre, F.; Audebert, P. Metal-Free Synthetic Approach to 3-Monosubstituted Unsymmetrical 1,2,4,5-Tetrazines Useful for Bioorthogonal Reactions. Angew. Chem., Int. Ed. 2018, 57, 12057–12061.
(18) Yang, J.; Carver, M. R.; Li, W.; Sahu, S.; Devaraj, N. K. Metal-Catalyzed One-Pot Synthesis of Tetrazines Directly from Aliphatic Nitriles and Hydrazine. Angew. Chem., Int. Ed. 2012, 51, 5222–5225.
(29) Xie, Y.; Fang, Y.; Huang, Z.; Tallon, A. M.; am Ende, C. W.; Fox, J. M. Divergent Synthesis of Monosubstituted and Unsymmetrical 3,6-Disubstituted Tetrazines from Carboxylic Ester Precursors. Angew. Chem., Int. Ed. 2020, 59, 16967−16973.

(30) Mao, W.; Shi, W.; Li, J.; Su, D.; Wang, X.; Zhang, L.; Pan, L.; Wu, X.; Wu, H. Organocatalytic and Scalable Syntheses of Unsymmetrical 1,2,4,5-Tetrazines by Thiol-Containing Promoters. Angew. Chem., Int. Ed. 2019, 58, 1106−1109.

(31) Lambert, W. D.; Fang, Y.; Mahapatra, S.; Huang, Z.; am Ende, C. W.; Fox, J. M. Installation of Minimal Tetrazines through Silver-Mediated Liebeskind–Srogl Coupling with Arylboronic Acids. J. Am. Chem. Soc. 2019, 141, 17068−17074.

(32) Liu, F.; Liang, Y.; Houk, K. N. Theoretical Elucidation of the Origins of Substituent and Strain Effects on the Rates of Diels−Alder Reactions of 1,2,4,5-Tetrazines. J. Am. Chem. Soc. 2014, 136, 11483−11493.

(33) Shin, Y.-H.; Koh, H.-J.; Um, I.-H. Kinetic Study on Alkaline Hydrolysis of 2-Pyridyl and 4-Pyridyl X-substituted Benzoates: Effects of Benzyol Substituent X and Leaving-Group Basicity on Reactivity and Reaction Mechanism. Bull. Korean Chem. Soc. 2017, 38, 1138−1142.

(34) Hansch, C.; Leo, A.; Taft, R. W. A survey of Hammett substituent constants and resonance and field parameters. Chem. Rev. 1991, 91, 165−195.