Design of Spiro[2.3]hex-1-ene, a Genetically Encodable Double-Strained Alkene for Superfast Photoclick Chemistry

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S *Supporting Information*

ABSTRACT: Reactive yet stable alkene reporters offer a facile route to studying fast biological processes via the cycloaddition-based bioorthogonal reactions. Here, we report the design and synthesis of a strained spirocyclic alkene, spiro[2.3]hex-1-ene (Sph), for an accelerated photoclick chemistry, and its site-specific introduction into proteins via amber codon suppression using the wild-type pyrrolysyl-tRNA synthetase/tRNA$_{CUA}$ pair. Because of its high ring strain and reduced steric hindrance, Sph exhibited fast reaction kinetics (k_2 up to 34 000 M^{-1} s^{-1}) in the photoclick chemistry and afforded rapid (<10 s) bioorthogonal protein labeling.
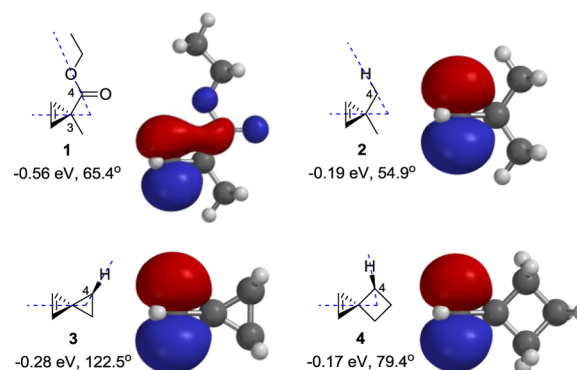
Figure 1. Structures of cyclopropene derivatives and their optimized geometries. The LUMO orbitals are rendered on the structures, with the LUMO energies indicated in eV. Calculations were performed using the B3LYP method at the 6-311++G** level in a vacuum at 298 K. The dihedral angles between the cyclopropene plane of symmetry line (in blue) and the indicated C^4−H or C^4−O bond are shown.

The use of strain, a classic concept in organic chemistry,[1] to accelerate bioorthogonal reactions has attracted a lot of interest recently, particularly in the cycloaddition reactions.[2] Some prominent examples include strain-promoted azide−alkyne cycloaddition,[3] nitrone−alkyne cycloaddition,[4] tetrazine−alkene cycloaddition,[5] and tetrazole−alkene cycloaddition ("photoclick chemistry").[6] With rare exceptions,[7] the majority of strained substrates serve as dipolarophiles or dienophiles, such as cyclooctyne and its variants,[3] thiacycloheptyne,[8] *trans*-cyclooctene,[5a] norbornene,[5b] and cyclopropene and its derivatives.[6b,9] To further enhance the reactivity of these strained substrates, additional conformational controls have been devised, such as the fusion of a second ring. For example, Fox and co-workers elegantly designed a cyclopropane-fused *trans*-cyclooctene that adopts the higher-energy "half-chair" conformation and affords a second-order rate constant, k_2, of 22 000 M^{-1} s^{-1} in the inverse electron-demand Diels−Alder reaction with 3,6-di(2-pyridyl)-*s*-tetrazine in methanol, 18 times faster than the parent *trans*-cyclooctene.[10] Similarly, van Delft and co-workers ingeniously fused the cyclopropane ring to cyclooctyne to generate bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN), which exhibited a rate acceleration of 70-fold (k_2 = 0.14 M^{-1} s^{-1}) over cyclooctyne in the nitrone−alkyne cycloaddition reaction.[11] A recent report by Chin and Fox et al. revealed that BCN is also an excellent substrate for the tetrazine ligation reaction with a 3,6-di(2-pyridyl)-*s*-tetrazine derivative (k_2 = 437 M^{-1} s^{-1}).[12]

We previously reported the synthesis of a stable and genetically encodable 3,3-disubstituted cyclopropene[6b] (**1** in Figure 1) as a privileged substrate for photoclick chemistry (k_2 = 58 M^{-1} s^{-1} with 2-(*p*-methoxyphenyl)-5-phenyltetrazole (**Tet-1**) in 1:1 acetonitrile/PBS). The small ring size forces C^3-substituents into close proximity with the cyclopropene π-bond,

which may cause considerable steric clash with the aryl substituents of the incoming nitrile imine along the reaction coordinate. Therefore, we envisioned that the cycloaddition reaction could be accelerated if we decrease this steric hindrance. Indeed, computational studies suggested that this type of steric hindrance is a major impediment in obtaining fast reaction kinetics in the dibenzocyclooctyne-mediated cyclo-addition reaction.[13] Here, we report the design, synthesis, and genetic encoding of spiro[2.3]hex-1-ene, an unprecedented stable cyclopropene derivative that showed superior reactivity in photoclick chemistry.

To relieve the steric repulsion, we considered four basic cyclopropene structures, as shown in Figure 1. DFT calculations indicated that the LUMO energies of these four structures followed the order of **1** < **3** < **2** ≈ **4**, suggesting that cyclopropene **1** has the smallest HOMO−LUMO gap, which may lead to the fastest cycloaddition reaction.[14] On the other hand, the addition of an exocyclic 3- or 4-membered ring (**3** and **4**) significantly altered the projections of the hydrogens at the C^4 position; the dihedral angles between the cyclopropene ring symmetry line and the C−H bond increased to 122.5° and 79.5°, respectively. Considering the size of H vs O, the steric hindrance presented by these four cyclopropene structures toward an incoming nitrile imine dipole should follow the order of **3** < **4** < **2** < **1**. Taking these factors together, the reactivity
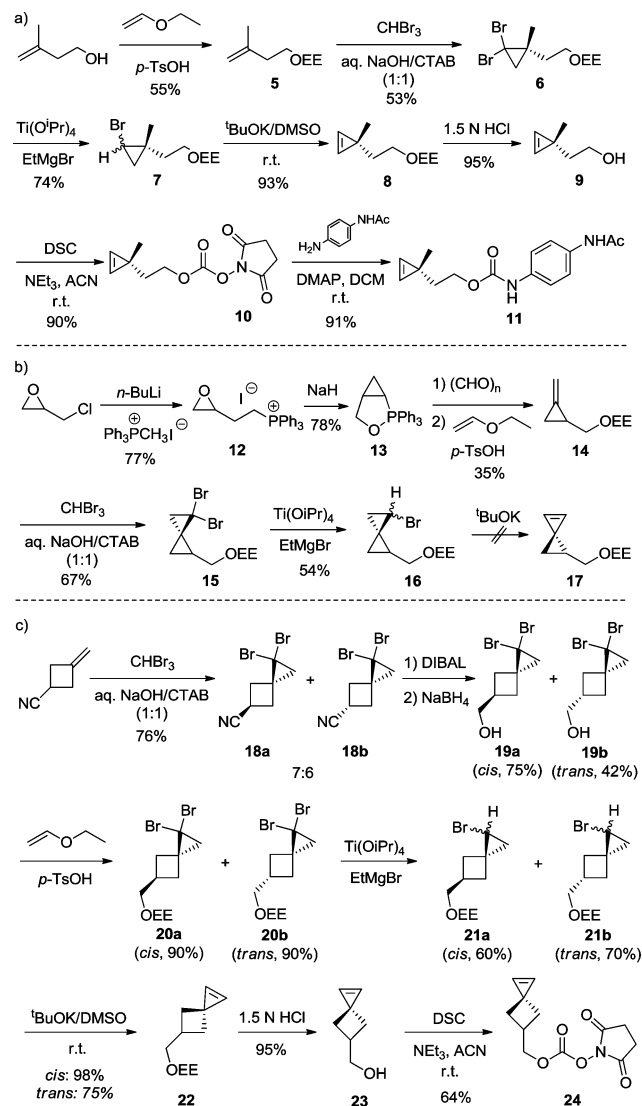
trend should follow the order of **3** > **4** > **2**, with the position of cyclopropene **1** uncertain because of the two opposing effects.

To experimentally determine the reactivity trend, we set out to prepare three new cyclopropene derivatives based on the structures of **2**–**4** (Scheme 1). The 3,3-dialkyl-substituted

Scheme 1. Synthesis of Cyclopropene Derivatives

cyclopropene **9** was obtained from ethyl-3-methylbut-3-enol (Scheme 1a) via the following key steps: (i) cyclopropanation with the *in situ*-generated dibromocarbene in the presence of phase-transfer catalyst cetyltrimethylammonium bromide (CTAB) to form dibromocyclopropane **6**; (ii) Ti(O^{i}Pr)$_4$-catalyzed mono-debromination to form bromocyclopropane **7**; and (iii) base-mediated elimination to generate the 3,3-dialkylated cyclopropene **8**. For X-ray structural determination, crystalline carbamate analogue **11** was also prepared. In parallel, the synthesis of spiro[2.2]pentene began with the ethoxyethyl-protected (methylenecyclopropyl)carbinol **14** (Scheme 1b), prepared using a published procedure.[15] Subsequent cyclopropanation and mono-debromination proceeded smoothly to afford a diastereomeric mixture of 1-bromo-spiro[2.2]pentane **16** in an overall yield of 36%. However, treatment of **16** with potassium *tert*-butoxide in DMSO did not produce the desired spiro[2.2]pentene **17** as reported,[16] presumably due to the high

strain energy (~90 kcal/mol) in this spirocyclic system.[17] The extensive search for an alternative base to effect the elimination was not successful, suggesting the spiropentene might be unstable at room temperature. In this regard, spiro[2.3]hexene should offer a balance between stability and reactivity because of its larger ring size. The synthesis of spiro[2.3]hex-1-ene **23** started with cyclopropanation of the commercially available 3-methylene-cyclobutanecarbonitrile to produce the diastereomers **18a** and **18b** in 7:6 ratio with a combined yield of 76% (Scheme 1c). These two diastereomers were separated and allowed to proceed in parallel in subsequent transformations: (i) reduction of the nitrile group to the alcohol through sequential treatments of DIBAL and NaBH$_4$; (ii) protection of the alcohol by ethyl vinyl ether; and (iii) mono-debromination to give mono-bromo-spirohexane **21a**/**21b** (Scheme 1c). To our satisfaction, the elimination reactions with **21a**/**21b** proceeded smoothly to generate spiro[2.3]hex-1-ene **22** with excellent yields. A succinimidyl carbonate derivative **24** was then prepared for the crystallographic study.

The crystal structures of cyclopropene **11** and spiro[2.3]hex-1-ene **24** were obtained (see Tables S1 and S2 in the Supporting Information (SI) for crystal data and structural refinement), allowing us to compare them with 3-methyl-3-cyclopropenecarboxylic acid that was determined previously[6b] (Figure 2). From the top view, the three cyclopropene rings are
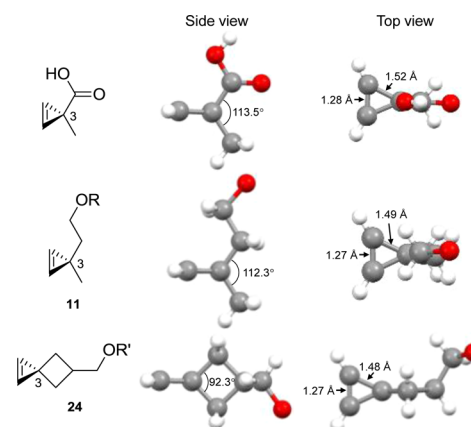

Figure 2. Crystal structures of the three cyclopropene derivatives viewed from the side and the top. The carbamate group in **11** and the carbonate group in **24** are omitted for clarity.

essentially identical, with the C=C bond length of 1.27–1.28 Å and the opposing bond angle of approximately 50°. However, from the side view the bond angle between two C^3-substituents decreased dramatically from 113.5° for 3-methyl-3-cyclopropenecarboxylic acid and 112.3° for **11** to 92.3° for spiro[2.3]hex-1-ene **24**. As expected, the cyclobutane ring in **24** pulls the C^3-substituents away from the π-faces of the cyclopropene ring, resulting in reduced steric hindrance.

To examine whether reduced steric hindrance in spiro[2.3]-hex-1-ene leads to faster cycloaddition reaction, we performed pairwise comparison studies in which a mixture of cyclopropenes were incubated with **Tet-1** in CD$_3$CN and competitive formation of the pyrazoline products was monitored by ^{1}H NMR (Figure 3).[18] The reactions proceeded cleanly in the NMR tube (Figures S4 and S5 in SI) upon photoirradiation with a hand-held 302 nm UV lamp (UVM, 0.16 A, 2.3 mW/cm^2). Based on the characteristic pyrazoline proton signals, spirohexene **22** was about 17 times more
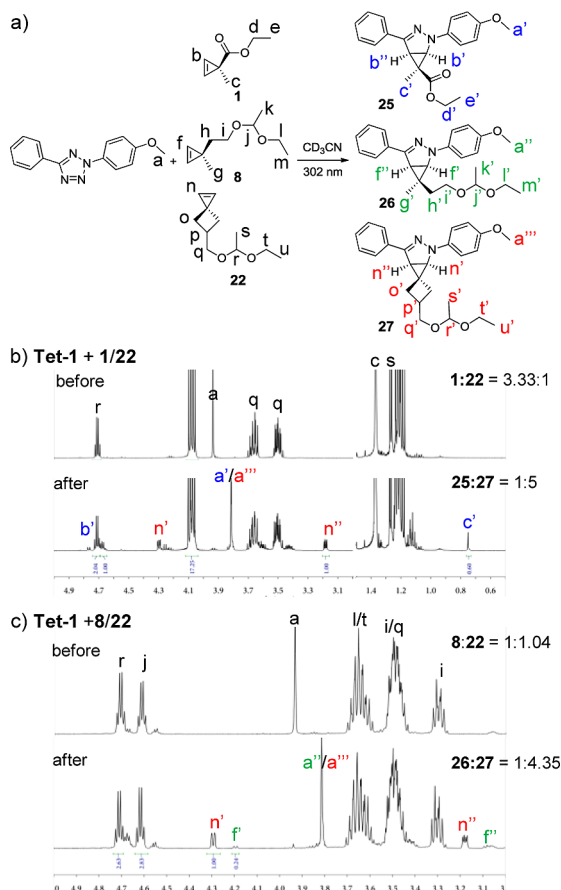
Figure 3. Competitive cycloadditions of **Tet-1** with pairs of cyclopropene dipolarophiles in CD₃CN at 25 °C. (a) Reaction scheme. (b,c) Selected regions of ¹H NMR of the reaction mixtures before and after 302 nm photoirradiation, showing the characteristic proton signals for pyrazoline **25–27**. See Figures S1–S5 in SI for spectrum assignment and ratio determination details.

Figure 4. M06-2X/6-311+G(d,p)-optimized transition-state structures for the cycloaddition of the nitrile imine with **1**, **2** (simplified **8**), and 5-methylspiro[2.3]hex-1-ene **22d** (simplified **22**) in water at 298 K. The bond lengths (in Å) at the saddle point are marked on the TS structures. The activation energies, $\Delta G^{\circ\ddagger}$, are in kcal/mol, and the single imaginary frequencies, ν_i, are in cm⁻¹. The relative rate constants, k_{rel}, were computed on the basis of $\Delta\Delta G^{\circ\ddagger}$.

reactive than cyclopropene **1** (Figure 3b) and 4 times more reactive than cyclopropene **8** (Figure 3c). A separate HPLC-based kinetic study of the cycloaddition of **Tet-1** with spirohexene **22** in PBS/ACN (1:1) gave a cycloaddition rate constant of 890 ± 51 M⁻¹ s⁻¹ (Figure S6 in SI), about 15 times faster than the reaction of cyclopropene **1** under the same condition.[6b] Furthermore, when Cl⁻-free phosphate buffer/ACN (1:1) was used as the solvent, the cycloaddition rate constant increased to 2600 ± 180 M⁻¹ s⁻¹ (Figure S7 in SI), consistent with a recent report that the nitrile imine–alkene cycloaddition is extremely fast in the absence of Cl⁻.[19] To understand the basis of the reactivity trend among the cyclopropene derivatives, we conducted a DFT-based search of the transition states (TSs) for the cycloaddition reactions (Figure 4).[20] We found that for cyclopropene **1** the exo TS is favored over the endo TS by 2.0 kcal/mol, presumably due to increased steric hindrance and lack of secondary interactions (a result of orthogonal arrangement of the nitrile imine π system and the carbonyl π system) in the endo TS. Compared with cyclopropene **1**, cyclopropene **8** and spirohexene **22** showed lower activation barriers, which led to 2.3 and 15 times faster cycloaddition reaction, respectively (Figure 4). These results agree well with the NMR-based competition studies (Figure 3).

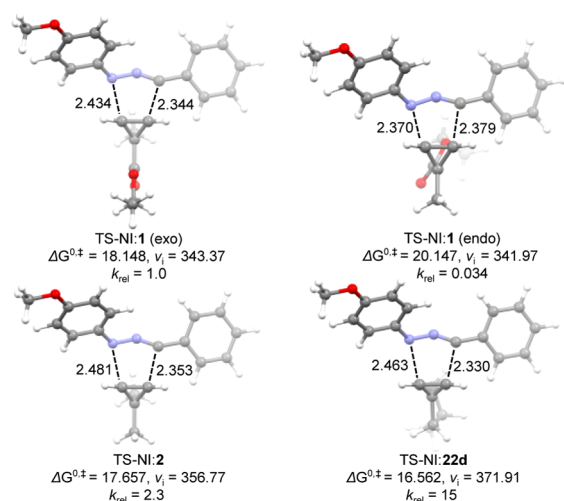Since the *Methanosarcina mazei* pyrrolysyl-tRNA synthetase (PylRS)/tRNA_CUA pair has shown tremendous versatility in genetically encoding structurally diverse lysine derivatives,[21] we suspected that spiro[2.3]hexene could be similarly introduced into proteins site-specifically using this system. Accordingly, we prepared N^{ε}-(spiro[2.3]hex-1-ene-5-methoxycarbonyl)-L-lysine (SphK) from **24** (Scheme S4 in SI) and found that SphK is stable toward excess glutathione (Figure S8 in SI). To our delight, SphK was efficiently incorporated into superfolder GFP (sfGFP) carrying an amber codon at position 2 in BL21(DE3) cells expressing the wild-type PylRS/tRNA_CUA pair. The SphK-encoded sfGFP (sfGFP-S2SphK) was purified in a yield of 4.9 mg/L (Figure S9 in SI). For comparison, the CpK-encoded sfGFP (sfGFP-S2CpK) was expressed at 4.1 mg/L when an engineered PylRS/tRNA_CUA pair previously reported to charge N^{ε}-acryloyl-L-lysine[22] was employed (Figure S10 in SI). Subsequent kinetic studies revealed that sfGFP-S2SphK reacted with **Tet-1** in PBS/CH₃CN (2:1) with $k_2 = 1850 \pm 218$ M⁻¹ s⁻¹, about 9 times faster than sfGFP-S2CpK ($k_2 = 206 \pm 17$ M⁻¹ s⁻¹) (Figure 5; Figures S11 and S12 in SI). To eliminate the inhibitory effect of Cl⁻, we also ran the reaction in the Cl⁻-free phosphate buffer/CH₃CN (2:1) and found k_2 to be 7000 ± 320 M⁻¹ s⁻¹, about 4 times faster than in Cl⁻-containing PBS/CH₃CN (2:1) buffer (Figure 5b; Figure S13 in SI). To completely remove CH₃CN from the solvent system, we synthesized a water-soluble **Tet-2** carrying a sulfonic acid group (Scheme S5 in SI; structure shown in Figure 5a). In the fluorescence-based verification study, **Tet-2** maintains excellent reactivity toward spirohexene **22** ($k_2 = 34\,000 \pm 1300$ M⁻¹ s⁻¹ in CH₃CN/phosphate buffer (1:1); Figure S14 in SI). When sfGFP-SphK was treated with **Tet-2** in phosphate buffer under identical photoirradiation conditions, the second-order rate constant, k_2, was determined to be 10 420 ± 810 M⁻¹ s⁻¹ (Figure 5b; Figure S15 in SI), in a range close to the tetrazine ligation ($k_2 = 22\,000$ M⁻¹ s⁻¹ for protein substrates as measured by a fluorescence-based assay).[10]

In summary, we have synthesized a biocompatible spirocyclic alkene reporter that is stable at ambient conditions and yet highly reactive toward tetrazoles in photoclick chemistry. Crystallographic analysis and computational studies indicated
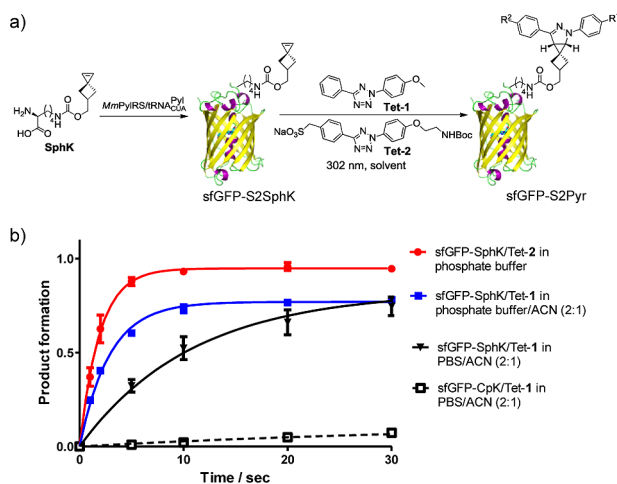
Figure 5. Comparison of the reactivity of CpK- and SphK-encoded sfGFP in photoclick chemistry. (a) Scheme for genetic incorporation of SphK into sfGFP and its subsequent reaction with the tetrazoles. (b) Plots of the photoclick reactions of sfGFP-S2CpK and sfGFP-S2SphK with **Tet-1** or **Tet-2** in various solvent systems. The reactions were set up by incubating 5 μM sfGFP-S2CpK or sfGFP-S2SphK and 50 μM **Tet-1**/**Tet-2** in PBS/ACN (2:1), phosphate buffer/ACN (2:1), or phosphate buffer only. The mixtures were irradiated with a 302 nm hand-held UV lamp for the indicated time prior to LC-MS analysis.

that the enhanced reactivity is due to the unique spirocyclic structure, which alleviates steric repulsion in the transition state in addition to the ring strain present in the cyclopropene. Moreover, a lysine derivative containing the spiro[2.3]hex-1-ene moiety was incorporated into proteins site-specifically using the amber codon suppression technique, which in turn directed fast (<10 s) and specific protein modification by a water-soluble tetrazole via the photoclick reaction with a k_2 value exceeding 10 000 M^{-1} s^{-1}. Exploitation of this genetically encodable, robust alkene reporter to study class B GPCR activation[23] in living cells is currently underway.

ASSOCIATED CONTENT

ⓢ Supporting Information

Supplemental figures and tables, synthetic schemes, experimental procedures, characterization of all new compounds, X-ray structural data, and computational results. These materials are available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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determination for compounds **11** and **24** (Cambridge Structural Database accession numbers CCDC 983641 and 983642, respectively).

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