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

Zhipeng Yu and Qing Lin*

Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260, United States

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<sub>CUA</sub> pair. Because of its high ring strain and reduced steric hindrance, Sph exhibited fast reaction kinetics (<10 s) in the photoclick chemistry and afforded rapid bioorthogonal protein labeling.

The use of strain, a classic concept in organic chemistry, to accelerate bioorthogonal reactions has attracted a lot of interest recently, particularly in the cycloaddition reactions. Some prominent examples include strain-promoted azide–alkyne cycloaddition, nitrene–alkyne cycloaddition, tetrazine–alkene cycloaddition, and tetrazole–alkene cycloaddition ("photoclick chemistry"). With rare exceptions, the majority of strained substrates serve as dipolarophiles or dienophiles, such as cyclooctyne and its variants, thiacycloheptyne, thiacyclooctyne, norbornene, and cyclopropene and its derivatives. 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<sub>2</sub>, of 22 000 M<sup>−1</sup> s<sup>−1</sup> 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. Similarly, van Delft and co-workers ingeniously fused the cyclopropene ring to cyclooctyne to generate bicyclo[6.1.0]non-4-yn-9-ylmethanol (BCN), which exhibited a rate acceleration of 70-fold (k<sub>2</sub> up to 34 000 M<sup>−1</sup> s<sup>−1</sup>) in the inverse electron-demand Diels–Alder reaction. 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 cyclopropene structures followed the order of C<sub>4</sub>-H vs C<sub>3</sub>-O bond. Considering the size of H vs O, the steric hindrance presented by these four cyclopropene structures, as shown in Figure 1. DFT calculations indicated that the LUMO energies of these four cyclopropene structures followed the order of C<sub>4</sub>-H vs C<sub>3</sub>-O bond. 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 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 cycloaddition reaction.

Received: February 5, 2014
Published: March 4, 2014
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 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(OPr)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. 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, presumably due to the high strain energy ($\sim$90 kcal/mol) in this spirocyclic system. 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 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 diastereomeres 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 NaBH₄; (ii) protection of the alcohol by ethyl vinyl ether; and (iii) mono-debromination to give mono-bromo-spirohexene 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-cyclopropencarboxylic acid that was determined previously (Figure 2). From the top view, the three cyclopropene rings are 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 C₃ substituents decreased dramatically from 113.5° for 3-methyl-3-cyclopropencarboxylic 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 C₃ 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 CD₃CN and competitive formation of the pyrazoline products was monitored by 1H NMR (Figure 3). 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/cm²). Based on the characteristic pyrazoline proton signals, spirohexene 22 was about 17 times more reactiv...
consistent with a recent report that the nitrile imine cycloaddition is extremely fast in the absence of Cl cyclopropene dipolarophiles in CD$_3$CN at 25°C (Figure 4).20 We found that for cyclopropene understand the basis of the reactivity trend among the 1 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 (PyrRS)/tRNA$^{\text{Clua}}$ 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 $N^\text{ε}$-(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 PyrRS/tRNA$^{\text{Clua}}$ 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 PyrRS/tRNA$^{\text{Clua}}$ pair previously reported to charge $N^\text{ε}$-acyrloyl-l-lysine$^{22}$ was employed (Figure S10 in SI). Subsequent kinetic studies revealed that sfGFP-S2SphK reacted with Tet-1 in PBS/CH$_3$CN (1:1) with $k_2 = 1850 ± 218$ M$^{-1}$ s$^{-1}$, about 9 times faster than sfGFP-S2Cpk ($k_2 = 206 ± 17$ 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/CH$_3$CN (2:1) buffer (Figure Sb; Figure S13 in SI). To completely remove CH$_3$CN 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 ($k_2 = 34 000 ± 1300$ M$^{-1}$ s$^{-1}$ in CH$_3$CN/phosphate buffer (1:1); Figure S10) in CH$_3$CN (2:1) buffer (Figure Sb; Figure S13 in SI). When sfGFP-SphK was treated with Tet-2 in phosphate buffer under identical photoligation conditions, the second-order rate constant, $k_2$, was determined to be 10 420 ± 810 M$^{-1}$ s$^{-1}$ (Figure Sb; Figure S15 in SI), in a range close to the tetracene 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 spirocyclc alkene reporter that is stable at ambient conditions and yet highly reactive toward tetracenes in photoclick chemistry. Crystallographic analysis and computational studies indicated

Figure 3. Competitive cycloadditions of Tet-1 with pairs of cyclopropene dipolarophiles in CD$_3$CN at 25°C. (a) Reaction scheme. (b,c) Selected regions of $^1$H 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+(d,p)-optimized transition-state structures for the cycloaddition of the nitrile imine with 1, 2 (simplified 8), and 5-methylenespiro[2.3]hex-1-ene 22 (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, Δ$\Delta$G°, are in kcal/mol, and the single imaginary frequencies, $\nu_i$, are in cm$^{-1}$. The relative rate constants, $k_2$, were computed on the basis of ΔΔG°.
that the enhanced reactivity is due to the unique spiropyrolic structure, which alleviates steric repulsion in the transition state in addition to the ring strain present in the cyclopropane. 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 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.

#### AUTHOR INFORMATION

**Corresponding Author**
qiniling@buffalo.edu

**Notes**

The authors declare no competing financial interest.

#### ACKNOWLEDGMENTS

We gratefully acknowledge the National Institutes of Health (GM 085092) and the National Science Foundation (CHE-1305826) for financial support. The FT-ICR mass spectrometer used in this study was supported through a grant from the NIH National Center for Research Resources (S10RR029517). We thank Prof. Wenshe Liu at Texas A&M University for generously providing us the plasmids pEvol-PyTT-PyTRS, pEvol-AcrKRS, and pET-sfGFPSTAG, and William Brennessel at the University of Rochester for X-ray structural determination for compounds 11 and 24 (Cambridge Structural Database accession numbers CCDC 983641 and 983642, respectively).

### REFERENCES

(1) Wiberg, K. B. Angew. Chem., Int. Ed. Engl. 1986, 25, 312–322.
(2) Ramil, C. P.; Lin, Q. Chem. Commun. 2013, 49, 11007–11022.
(3) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. J. Am. Chem. Soc. 2004, 126, 15046–15047. (b) Sletten, E. M.; Bertozzi, C. R. Acc. Chem. Res. 2011, 44, 666–676. (c) Ning, X.; Guo, J.; Wollert, M. A.; Boons, G. J. Angew. Chem. Int. Ed. 2008, 47, 2253–2255.
(4) (a) Ning, X.; Temming, R. P.; Dommerholt, J.; Guo, J.; Ania, D. B.; Debets, M. P.; Wollert, M. A.; Boons, G. J.; van Delft, F. L. Angew. Chem., Int. Ed. 2010, 49, 3065–3068. (b) McKay, C. S.; Moran, J.; Pezacki, J. P. Chem. Commun. 2010, 46, 931–933.
(5) (a) Blackman, M. L.; Rozen, M.; Fox, J. M. J. Am. Chem. Soc. 2008, 130, 13518–13519. (b) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. Bioconjugate Chem. 2008, 19, 2297–2299.
(6) (a) Lim, R. K. V.; Lin, Q. Acc. Chem. Res. 2011, 44, 828–839. (b) Yu, Z.; Pan, Y.; Wang, Z.; Wang, J.; Lin, Q. Angew. Chem. Int. Ed. 2012, 51, 10600–10604.
(7) (a) Yu, Z.; Lim, R. K. V.; Lin, Q. Chem.— Eur. J. 2010, 16, 13315–13329. (b) McKay, C. S.; Blake, J. A.; Cheng, J.; Danielson, D. C.; Pezacki, J. P. Chem. Commun. 2011, 47, 10040–10042.
(8) de Almeida, G.; Sletten, E. M.; Nakamura, H.; Palaniappan, K. K.; Bertozzi, C. R. Angew. Chem., Int. Ed. 2012, 51, 2443–2447.
(9) (a) Yang, J.; Šeckuté, J.; Cole, C. M.; Devaraj, N. K. Angew. Chem., Int. Ed. 2012, 51, 7476–7479. (b) Patterson, D. M.; Nazarova, L. A.; Xie, B.; Kamber, D. N.; Prescher, J. A. J. Am. Chem. Soc. 2012, 134, 18638–18643. (c) Kamber, D. N.; Nazarova, L. A.; Liang, Y.; Lopez, S. A.; Patterson, D. M.; Shih, H. W.; Houk, K. N.; Prescher, J. A. J. Am. Chem. Soc. 2013, 135, 13680–13683.
(10) Taylor, M. T.; Blackman, M. L.; Dimitrenko, O.; Fox, M. J. Am. Chem. Soc. 2011, 133, 9646–9649.
(11) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, F.; van Delft, F. L. Angew. Chem., Int. Ed. 2010, 49, 9422–9425.
(12) Lang, K.; Davis, L.; Wallace, S.; Mahesh, M.; Cox, D. J.; Blackman, M. L.; Fox, J. M.; Chin, J. W. J. Am. Chem. Soc. 2012, 134, 10317–10320.
(13) Chenoweth, K.; Chenoweth, D.; Goddard, W. A., III Org. Biomol. Chem. 2009, 7, 5255–5258.
(14) Wang, Y.; Song, W.; Hu, W. J.; Lin, Q. Angew. Chem., Int. Ed. 2009, 48, 5330–5333.
(15) Okuma, K.; Tanaka, Y.; Yoshihara, K.; Ezaki, A.; Koda, G.; Ohla, H. J. Org. Chem. 1993, 58, 5915–5917.
(16) Bloch, R.; Denis, J. -M. Angew. Chem., Int. Ed. 1980, 19, 928–929. Spiropentene was obtained as a solution in chloroform after the elimination reaction under low pressure (80 Torr). In condensed phase even at ~78°C, spiropentene was found to polymerize.
(17) Kao, J.; Radom, L. J. Am. Chem. Soc. 1978, 100, 760–767.
(18) Initial studies of the individual cycloaddition reactions by 'H NMR indicated that the rates of cycloaddition rate were similar, which can be attributed to the fact that at high concentrations (~50 mM) the tetrazole ring-nutation became the rate-determining step. See Figures S1–S3 in SI for details.
(19) Wang, X. S.; Lee, Y. J.; Liu, W. R. Chem. Commun. 2014, 50, 3176–3179.
(20) See Supporting Information for computational details.
(21) (a) Nguyen, D. P.; Lusic, H.; Neumann, H.; Kapadnis, P. B.; Deiters, A.; Chin, J. W. J. Am. Chem. Soc. 2009, 131, 8720–8721. (b) Lang, K.; Davis, L.; Torres-Kolbus, J.; Chou, C.; Deiters, A.; Chin, J. W. Nat. Chem. 2012, 4, 298–304. (c) Wang, Y. S.; Fang, X.; Wallace, A. L.; Wu, B.; Liu, W. R. J. Am. Chem. Soc. 2012, 134, 2950–2953.
(22) Lee, Y. J.; Wu, B.; Raymond, J. E.; Zeng, Y.; Fang, X.; Wooley, K. L.; Liu, W. R. ACS Chem. Biol. 2013, 8, 1664–1670.
(23) Dong, M.; Koole, C.; Wootten, D.; Sexton, P. M.; Miller, L. J. Br. J. Pharmacol. 2014, 171, 1085–1101.