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Control and Design of Mutual Orthogonality in Bioorthogonal Cycloadditions

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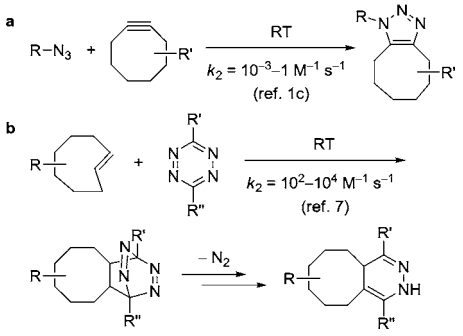
Supporting Information

ABSTRACT: The azide–dibenzocyclooctyne and *trans*-cyclooctene–tetrazine cycloadditions are both bioorthogonal and mutually orthogonal: *trans*-cyclooctene derivatives greatly prefer to react with tetrazines rather than azides, while dibenzocyclooctyne derivatives react with azides but not with tetrazines under physiological conditions. DFT calculations used to identify the origins of this extraordinary selectivity are reported, and design principles to guide discovery of new orthogonal cycloadditions are proposed. Two new bioorthogonal reagents, methylcyclopropene and 3,3,6,6-tetramethylthiacycloheptyne, are predicted to be mutually orthogonal in azide and tetrazine cycloadditions.

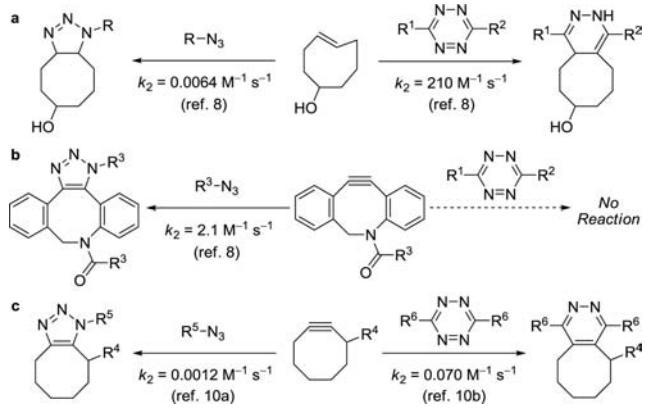
Azide and tetrazine cycloadditions have become central reactions in the rapidly developing field of cellular component labeling with bioorthogonal reactions.[1−3] Bertozzi and co-workers have developed strain-promoted (3 + 2) cycloaddition reactions between azides and cyclooctynes since 2004 (Scheme 1a).[4] These reactions proceed at a rate that is

Scheme 1. (a) Azide–Cyclooctyne (3 + 2) Cycloaddition and (b) *trans*-Cyclooctene–Tetrazine (4 + 2) Cycloaddition



sufficient for in vivo labeling without the toxic copper(I) catalysts traditionally employed in "click chemistry" involving azide cycloadditions. Several groups have developed structurally varied cyclooctyne derivatives with different chemical reactivities and physical properties.[5] Another breakthrough in this area came in 2008 with the application of inverse-electron-demand Diels–Alder reactions of 1,2,4,5-tetrazines and strained alkenes (Scheme 1b).[6] In particular, the *trans*-cyclooctene–tetrazine (4 + 2) cycloaddition, which has an extremely high bimolecular rate constant (k_2 = 10^2–10^4 M^{-1} s^{-1}),[7] is much faster than the azide–cyclooctyne (3 + 2) cycloaddition (k_2 = 10^{-3}–1 M^{-1} s^{-1}).[1c] Recently, Hilderbrand and co-workers demonstrated that two bioorthogonal cycloaddition pairs are mutually orthogonal.[8] That is, as shown in Scheme 2a, *trans*-

Scheme 2. Selectivity of Bioorthogonal Cycloaddition Reactions[a]



[a]R–N_3 = Alexa Fluor 647 azide, R^1 = Me, R^2 = $(CH_2)_5NH_2$, R^3 = PEG4-CO_2H, R^4 = CH_2Ph-(*p*-CO_2H) (for azide cycloaddition) or H (for tetrazine cycloaddition), R^5 = Bn, and R^6 = Ph.

cyclooctene derivatives greatly prefer to react with tetrazines rather than azides, while dibenzocyclooctyne derivatives react with azides but not with tetrazines under physiological conditions (Scheme 2b). On the basis of this discovery, Hilderbrand and co-workers successfully realized the simultaneous labeling and imaging of two different cancer cell types in biological environments.[8] At almost the same time, Schultz, Lemke, and co-workers found that *trans*-cyclooctenes show extremely high selectivity toward tetrazines rather than azides in protein labeling experiments.[9] However, the cyclooctyne-modified proteins couple with both tetrazine-functionalized and azide-functionalized dyes.[9] The similar reactivities of cyclooctynes with azides and tetrazines was also demonstrated in separate kinetic studies by the Bertozzi and Wang groups: tetrazines react with cyclooctynes only 1–2 orders of magnitude faster than azides do (Scheme 2c).[10] *trans*-Cyclooctene, cyclooctyne, and dibenzocyclooctyne are all highly strained molecules; why do their selectivities toward azides and tetrazines under bioorthogonal cycloadditions differ

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dramatically? Here we answer this question using density functional theory (DFT) calculations, point out the factors that control the reactivity patterns of azides and tetrazines, develop a set of design principles to guide the discovery of new orthogonal cycloadditions, and predict that two new bioorthogonal reagents, methylcyclopropene and 3,3,6,6-tetramethylthiacycloheptyne, should be mutually orthogonal in azide and tetrazine cycloadditions.

We explored the cycloaddition reactions of *trans*-cyclooctene, cyclooctyne, and dibenzocyclooctyne with both methyl azide (**TS1**, **-3**, and **-5**, respectively) and dimethyltetrazine (**TS2**, **-4**, and **-6**, respectively) using DFT calculations.[11] M06-2X,[12] a density functional that we have shown to give relatively accurate energies for cycloadditions,[13] was used in this computational study. Figure 1 shows the transition-state structures **TS1**–**6** for the six investigated cycloaddition reactions. We also analyzed the activation barriers of these reactions using the distortion/interaction model,[14] in which the activation energy (E_{act}) is analyzed in terms of the distortion energy (E_{dist}) required for the geometrical deformation of the reactants to achieve their transition-state conformations and the interaction energy (E_{int}) arising from the interactions between the two distorted reactants in the transition state. The computed activation free energies, relative rate constants, and distortion/interaction energies are summarized in Table 1.

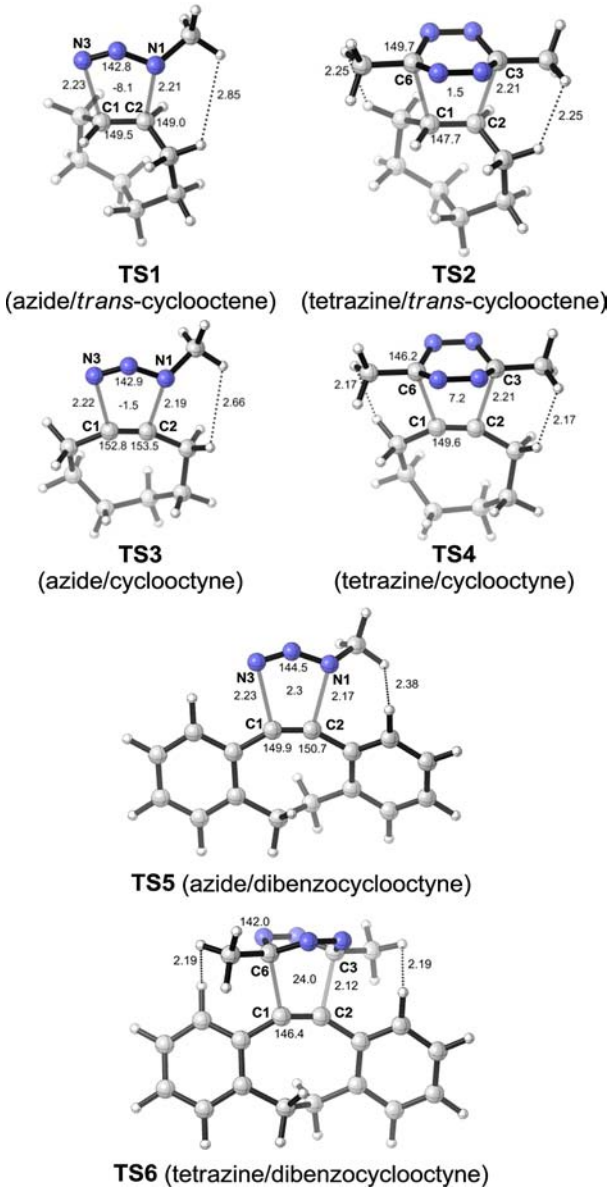

Figure 1. M06-2X/6-311+G(d,p)-optimized transition-state structures for cycloadditions with methyl azide (**TS1**, **-3**, and **-5**) and dimethyltetrazine (**TS2**, **-4**, and **-6**) (distances in Å, angles or dihedral angles in deg).

Table 1. M06-2X/6-311+G(d,p)-Computed Activation Free Energies in the Gas Phase and in Water (G_{gas} and G_{water}, in kcal/mol); Relative Rate Constants (k_{rel}, based on G_{water} at 298 K); and Activation, Distortion, and Interaction Energies (E_{act}, E_{dist}, and E_{int}, in kcal/mol)

	G_{gas}	G_{water}	k_{rel}	E_{act}	E_{dist}	E_{int}
TS1	25.0	26.4	2.0	12.3	20.5 (17.8[a])	−8.2
TS2	18.6	17.9	3.4×10^6	2.1	19.9 (16.4[b])	−17.8
TS3	25.0	26.8	1.0	11.7	20.6 (17.9[a])	−8.9
TS4	24.5	24.2	81	8.0	26.0 (20.3[b])	−18.0
TS5	21.9	23.9	1.3×10^2	7.7	20.4 (17.1[a])	−12.7
TS6	31.4	33.4	1.4×10^{-5}	13.7	36.7 (27.8[b])	−23.0
TS7	28.7	29.5	1.0×10^{-2}	15.0	24.5 (17.0[a])	−9.5
TS8	21.8	21.6	6.5×10^3	5.1	21.3 (12.5[b])	−16.2
TS9	19.7	21.7	5.5×10^3	5.7	15.6 (14.3[a])	−9.9
TS10	30.9	31.7	2.5×10^{-4}	13.2	34.4 (25.6[b])	−21.2

[a]Distortion energy of methyl azide. [b]Distortion energy of dimethyltetrazine.

trans-Cyclooctene, cyclooctyne, and dibenzocyclooctyne are all highly reactive because their distortion energies (3–6 kcal/mol, **TS1**–**5**) are much lower than those for unstrained alkenes or alkynes (8–17 kcal/mol).[14j,k]

For the cycloadditions of *trans*-cyclooctene, the activation free energy in water with tetrazine (via transition state **TS2**) is lower than that with azide (via **TS1**) by more than 8 kcal/mol (Table 1). This accounts for the almost exclusive tetrazine selectivity of *trans*-cyclooctenes in the experiments.[8,9] The distortion/interaction model analysis showed that the distortion energies of transition states **TS1** and **TS2** are nearly identical but that the favorable interaction energy of **TS2** is much larger than that of **TS1** (−17.8 vs −8.2 kcal/mol; Table 1). The different electronic properties of tetrazine and azide produce this large difference in the interaction energies. Frontier molecular orbital (FMO) analysis (Figure 2) indicated that the preferred orbital interaction is between the HOMO of

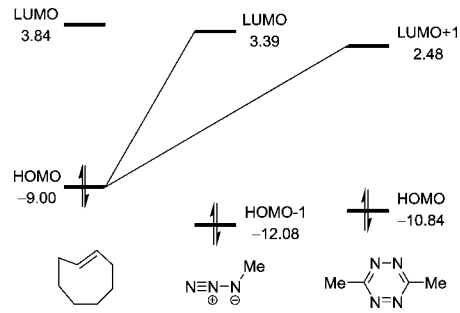

Figure 2. FMO diagram for the cycloadditions of *trans*-cyclooctene with methyl azide and dimethyltetrazine. HF//M06-2X/6-311+G(d,p)-computed orbital energies in eV are shown.

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trans-cyclooctene and the LUMO of methyl azide or a relevant vacant orbital of dimethyltetrazine.[15] Notably, azide is a much weaker electron acceptor than tetrazine because of its higher LUMO energy (3.39 vs 2.48 eV; Figure 2). The smaller orbital energy gap between *trans*-cyclooctene and tetrazine makes the favorable orbital interaction in **TS2** stronger than that in **TS1**. Therefore, tetrazines are much more reactive than azides in the cycloadditions using *trans*-cyclooctenes because of their higher electrophilicity.[16]

However, in the cycloadditions of dibenzocyclooctyne, dimethyltetrazine reacts 7 orders of magnitude slower than methyl azide (**TS5** and -**6**; Table 1), in good agreement with the experimental observation that dibenzocyclooctyne derivatives react only with azides.[8] The extremely sluggish kinetics of the dibenzocyclooctyne−tetrazine cycloaddition is mainly due to very high distortion energy for this reaction (36.7 kcal/mol for **TS6**). The structure of transition state **TS6** (Figure 1) shows that two distances between the methyl hydrogen atoms of tetrazine and the ortho hydrogen atoms of the aromatic rings of dibenzocyclooctyne are 2.19 Å, which is close to the sum of their van der Waals radii (2.20 Å).[17] This is achieved at the expense of increased distortions of the transition state and poor orbital overlap, as evidenced by the C3−C6−C1−C2 dihedral angle of 24° in **TS6**. The effects of the unfavorable steric repulsion and the poor orbital overlap greatly move the transition state **TS6** later along the reaction coordinate. A later transition state means a greater geometrical deformation of reactants, requiring more distortion energy. These factors are shown in the space-filling models of the reactants and transition states (Figure 3).[18] By contrast, the shortest H−H distance

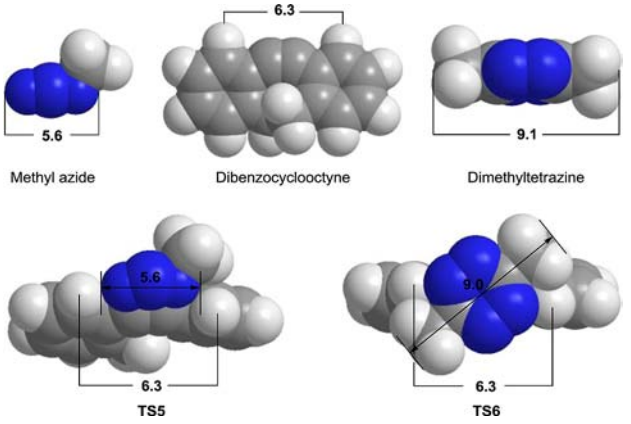


Figure 3. Space-filling models of dibenzocyclooctyne, methyl azide, dimethyltetrazine, and transition states **TS5** and **TS6** (distances in Å).

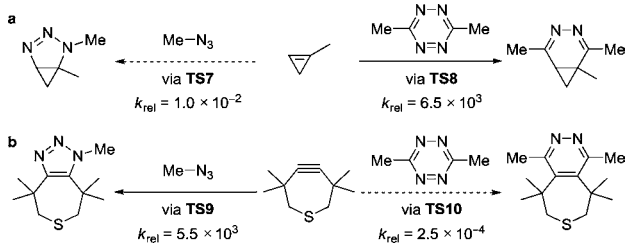
between methyl azide and dibenzocyclooctyne in **TS5** is 2.38 Å (Figure 1), implying that steric repulsions may be ignored. Moreover, the N1−N3−C1−C2 dihedral angle in **TS5** is 2.3°, and such a planar geometry ensures the maximum orbital overlap in the cycloaddition transition state. Although the electrophilicity of tetrazine is significantly higher than that of azide, the size of 3,6-disubstituted tetrazines is obviously larger than that of azide. In the case of dibenzocyclooctynes, because of the great steric hindrance caused by the two aryl hydrogen atoms ortho to the alkyne moiety, the steric effect overwhelms the electronic effect, leading to the exclusive azide selectivity.

For the cycloadditions of cyclooctyne, the activation free energy for the tetrazine (4 + 2) reaction (via transition state **TS4**) is 2.6 kcal/mol lower in water than that for the azide (3 + 2) reaction (via **TS3**) (Table 1). This indicates that the cyclooctyne−tetrazine cycloaddition is only a few orders of magnitude faster than the cyclooctyne−azide cycloaddition. The interaction energy of **TS4** is 9.1 kcal/mol greater in magnitude than that of **TS3** (−18.0 vs −8.9 kcal/mol) because of the favorable electronic effect of tetrazine, but the distortion energy of **TS4** is 5.4 kcal/mol higher than that of **TS3** (26.0 vs 20.6 kcal/mol) because of steric repulsions between dimethyltetrazine and the propargylic hydrogen atoms of cyclooctyne in **TS4** (Figure 1).

We can now propose generalized principles for the design of orthogonal reaction pairs in cycloadditions of the same electron-demand type.[19] The electronically more reactive electrophile (or nucleophile) **A** must be sterically more encumbered than the electronically less reactive one **B** (e.g, **A** = dimethyltetrazine, **B** = methyl azide). **A** reacts more readily with sterically unencumbered cycloaddition partners, but **B** reacts more readily with sterically encumbered ones.

We have used these principles to predict that two new bioorthogonal reagents, methylcyclopropene[3f] and 3,3,6,6-tetramethylthiacycloheptyne,[2c] should also be mutually orthogonal in azide and tetrazine cycloadditions (Scheme 3; the

Scheme 3. Prediction of Mutual Orthogonality of Two New Bioorthogonal Reagents in Azide and Tetrazine Cycloadditions



relative rate constants shown are predicted for aqueous solution). Methylcyclopropene derivatives show high rates of reaction with tetrazines,[3f,20] while 3,3,6,6-tetramethylthiacycloheptyne has been found to react readily with azides.[2c] The sterically encumbered but electronically reactive tetrazine should react much faster than the azide with the sterically unencumbered cyclopropene (Scheme 3a), while the azide should be much more reactive with the sterically encumbered cycloalkyne with four methyl groups adjacent to the alkyne moiety (Scheme 3b). The computed activation free energies, relative rate constants, and distortion/interaction energies of the corresponding cycloadditions further support our prediction (**TS7**−**10**; Table 1).[21] Further computational design of new bioorthogonal and orthogonal cycloadditions is ongoing in our laboratory.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Computational details and complete ref 11 (as SI ref 1). This material is 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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■ REFERENCES

(1) For reviews, see: (a) Sletten, E. M.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2009**, *48*, 6974. (b) Jewett, J. C.; Bertozzi, C. R. *Chem. Soc. Rev.* **2010**, *39*, 1272. (c) Debets, M. F.; van Berkel, S. S.; Dommerholt, J.; Dirks, A. J.; Rutjes, F. P. J. T.; van Delft, F. L. *Acc. Chem. Res.* **2011**, *44*, 805. (d) Devaraj, N. K.; Weissleder, R. *Acc. Chem. Res.* **2011**, *44*, 816.

(2) For recent examples of the azide cycloaddition, see: (a) Mbua, N. E.; Guo, J.; Wolfert, M. A.; Steet, R.; Boons, G.-J. *ChemBioChem* **2011**, *12*, 1912. (b) Plass, T.; Milles, S.; Koehler, C.; Schultz, C.; Lemke, E. A. *Angew. Chem., Int. Ed.* **2011**, *50*, 3878. (c) de Almeida, G.; Sletten, E. M.; Nakamura, H.; Palaniappan, K. K.; Bertozzi, C. R. *Angew. Chem., Int. Ed.* **2012**, *51*, 2443. (d) Hudak, J. E.; Barfield, R. M.; de Hart, G. W.; Grob, P.; Nogales, E.; Bertozzi, C. R.; Rabuka, D. *Angew. Chem., Int. Ed.* **2012**, *51*, 4161.

(3) For recent examples of the tetrazine cycloaddition, see: (a) Taylor, M. T.; Blackman, M. L.; Dmitrenko, O.; Fox, J. M. *J. Am. Chem. Soc.* **2011**, *133*, 9646. (b) Liu, D. S.; Tangpeerachaikul, A.; Selvaraj, R.; Taylor, M. T.; Fox, J. M.; Ting, A. Y. *J. Am. Chem. Soc.* **2012**, *134*, 792. (c) Seitchik, J. L.; Peeler, J. C.; Taylor, M. T.; Blackman, M. L.; Rhoads, T. W.; Cooley, R. B.; Refakis, C.; Fox, J. M.; Mehl, R. A. *J. Am. Chem. Soc.* **2012**, *134*, 2898. (d) 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. (e) Lang, K.; Davis, L.; Torres-Kolbus, J.; Chou, C.; Deiters, A.; Chin, J. W. *Nat. Chem.* **2012**, *4*, 298. (f) Yang, J.; Seckute, J.; Cole, C. M.; Devaraj, N. K. *Angew. Chem., Int. Ed.* **2012**, *51*, 7476.

(4) (a) Agard, N. J.; Prescher, J. A.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2004**, *126*, 15046. (b) Sletten, E. M.; Bertozzi, C. R. *Acc. Chem. Res.* **2011**, *44*, 666.

(5) (a) Baskin, J. M.; Prescher, J. A.; Laughlin, S. T.; Agard, N. J.; Chang, P. V.; Miller, I. A.; Lo, A.; Codelli, J. A.; Bertozzi, C. R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 16793. (b) Codelli, J. A.; Baskin, J. M.; Agard, N. J.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2008**, *130*, 11486. (c) Ning, X. H.; Guo, J.; Wolfert, M. A.; Boons, G. J. *Angew. Chem., Int. Ed.* **2008**, *47*, 2253. (d) Jewett, J. C.; Sletten, E. M.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2010**, *132*, 3688. (e) Dommerholt, J.; Schmidt, S.; Temming, R.; Hendriks, L. J. A.; Rutjes, F. P. J. T.; van Hest, J. C. M.; Lefeber, D. J.; Friedl, P.; van Delft, F. L. *Angew. Chem., Int. Ed.* **2010**, *49*, 9422. (f) Debets, M. F.; van Berkel, S. S.; Schoffelen, S.; Rutjes, F. P. J. T.; van Hest, J. C. M.; van Delft, F. L. *Chem. Commun.* **2010**, *46*, 97.

(6) (a) Blackman, M. L.; Royzen, M.; Fox, J. M. *J. Am. Chem. Soc.* **2008**, *130*, 13518. (b) Devaraj, N. K.; Weissleder, R.; Hilderbrand, S. A. *Bioconjugate Chem.* **2008**, *19*, 2297.

(7) Karver, M. R.; Weissleder, R.; Hilderbrand, S. A. *Bioconjugate Chem.* **2011**, *22*, 2263.

(8) Karver, M. R.; Weissleder, R.; Hilderbrand, S. A. *Angew. Chem., Int. Ed.* **2012**, *51*, 920.

(9) Plass, T.; Milles, S.; Koehler, C.; Szymanski, J.; Mueller, R.; Wiebler, M.; Schultz, C.; Lemke, E. A. *Angew. Chem., Int. Ed.* **2012**, *51*, 4166.

(10) (a) Agard, N. J.; Baskin, J. M.; Prescher, J. A.; Lo, A.; Bertozzi, C. R. *ACS Chem. Biol.* **2006**, *1*, 644. (b) Chen, W.; Wang, D.; Dai, C.; Hamelberg, D.; Wang, B. *Chem. Commun.* **2012**, *48*, 1736.

(11) Frisch, M. J.; et al. *Gaussian 09*, revision C.01; Gaussian, Inc.: Wallingford, CT, 2010.

(12) (a) Zhao, Y.; Truhlar, D. G. *Theor. Chem. Acc.* **2008**, *120*, 215. (b) Zhao, Y.; Truhlar, D. G. *Acc. Chem. Res.* **2008**, *41*, 157.

(13) (a) Paton, R. S.; Mackey, J. L.; Kim, W. H.; Lee, J. H.; Danishefsky, S. J.; Houk, K. N. *J. Am. Chem. Soc.* **2010**, *132*, 9335. (b) Lan, Y.; Zou, L.; Cao, Y.; Houk, K. N. *J. Phys. Chem. A* **2011**, *115*, 13906.

(14) (a) Ess, D. H.; Houk, K. N. *J. Am. Chem. Soc.* **2007**, *129*, 10646. (b) Ess, D. H.; Houk, K. N. *J. Am. Chem. Soc.* **2008**, *130*, 10187. (c) Hayden, A. E.; Houk, K. N. *J. Am. Chem. Soc.* **2009**, *131*, 4084. (d) Schoenebeck, F.; Ess, D. H.; Jones, G. O.; Houk, K. N. *J. Am. Chem. Soc.* **2009**, *131*, 8121. (e) van Zeist, W.-J.; Bickelhaupt, F. M. *Org. Biomol. Chem.* **2010**, *8*, 3118. (f) Fernández, I.; Cossío, F. P.; Bickelhaupt, F. M. *J. Org. Chem.* **2011**, *76*, 2310. (g) Lan, Y.; Wheeler, S. E.; Houk, K. N. *J. Chem. Theory Comput.* **2011**, *7*, 2104. (h) Paton, R. S.; Kim, S.; Ross, A. G.; Danishefsky, S. J.; Houk, K. N. *Angew. Chem., Int. Ed.* **2011**, *50*, 10366. (i) Fernández, I.; Bickelhaupt, F. M. *J. Comput. Chem.* **2012**, *33*, 509. (j) Gordon, C. G.; Mackey, J. L.; Jewett, J. C.; Sletten, E. M.; Houk, K. N.; Bertozzi, C. R. *J. Am. Chem. Soc.* **2012**, *134*, 9199. (k) Lopez, S. A.; Houk, K. N. *J. Org. Chem.* **2012**, DOI: 10.1021/jo301267b.

(15) In the cycloadditions of strained alkynes, FMO analysis showed that charge transfer from the alkyne to methyl azide or dimethyltetrazine also occurs. For details, see Figure S1 in the Supporting Information.

(16) Houk, K. N. *Acc. Chem. Res.* **1975**, *8*, 361.

(17) Rowland, R. S.; Taylor, R. *J. Phys. Chem.* **1996**, *100*, 7384.

(18) As shown in Figure 3, the two ortho hydrogen atoms of the aromatic rings in dibenzocyclooctyne form a 6.3 Å wide "gate" in the front of the carbon−carbon triple bond. Because the methyl group is not aligned with the two reacting nitrogen atoms of the azide, the effective size of methyl azide is 5.6 Å. Such a size can be accepted well by the 6.3 Å wide gate of dibenzocyclooctyne. In contrast, the two methyl groups and two reacting carbon atoms of the tetrazine are all in a straight line, so the effective size of dimethyltetrazine is up to 9.1 Å. Obviously, the 6.3 Å wide gate of dibenzocyclooctyne is so narrow that dimethyltetrazine has to twist to react.

(19) A second design principle is also being investigated: two cycloadditions with different electron demand (for example, the normal Diels−Alder reaction between a nucleophilic diene and an electrophilic dienophile and the inverse-electron-demand reaction of a nucleophilic dienophile and an electrophilic diene) are mutually orthogonal because of pure electronic effects.

(20) Thalhammer, F.; Wallfahrer, U.; Sauer, J. *Tetrahedron Lett.* **1990**, *31*, 6851.

(21) The activation free energy for the generation of the regioisomer (via **TS7a**) from methylcyclopropene and methyl azide in water is 29.7 kcal/mol. The structures of **TS7−10** and **TS7a** are given in Figure S2.