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Strain-promoted double-click reaction for chemical modification of azido-biomolecules†

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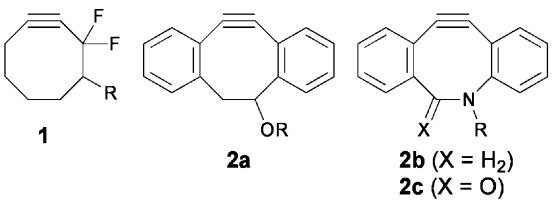
The strain-promoted "double-click" (SPDC) reaction using Sondheimer diyne, a novel convergent method conjugating three molecules spontaneously, has enabled us to readily modify an azido-biomolecule with a small reporter azido-molecule.

"Click reaction," copper(I)-catalyzed azide–alkyne [3+2] cycloaddition (CuAAC), is an emerging method for conjugating molecules in the fields of chemistry and biology.[1–3] With the progress in preparation techniques of biomolecules incorporated with bioorthogonal groups, various chemically-modified biomolecules have become available.[4] However, cytotoxicity by the copper catalyst and the slow rate of the reaction have restricted its application. To overcome these limitations, Bertozzi has introduced a copper-free click reaction, the strain-promoted azide–alkyne [3+2] cycloaddition (SPAAC),[5] exploiting the spontaneous reactivity of cyclooctynes toward an azide by its ring strain.[6] Furthermore, a rapid SPAAC reaction has been achieved using difluorinated cyclooctyne (DIFO, **1**),[7] dibenzocyclooctynol (DIBO, **2a**)[8] and aza-dibenzocyclooctyne (DIBAC, **2b**;[9] BARAC, **2c**[7f]) derivatives. Fluorescence-labeled or biotinylated derivatives of these cyclooctynes have enabled us to visualize the distribution of azido-glycoconjugates in cultured cells and in living animals.[7,8]



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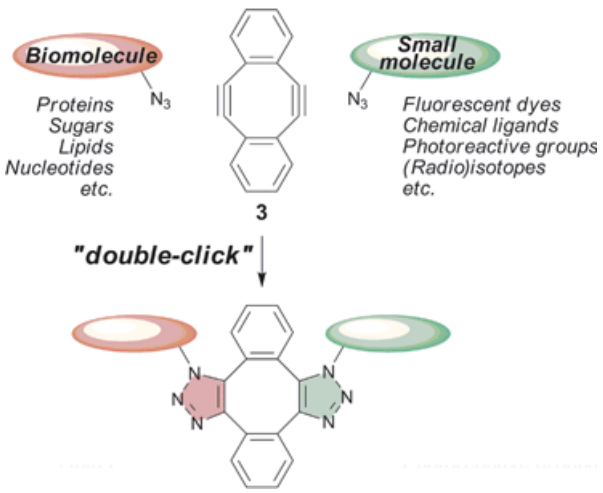
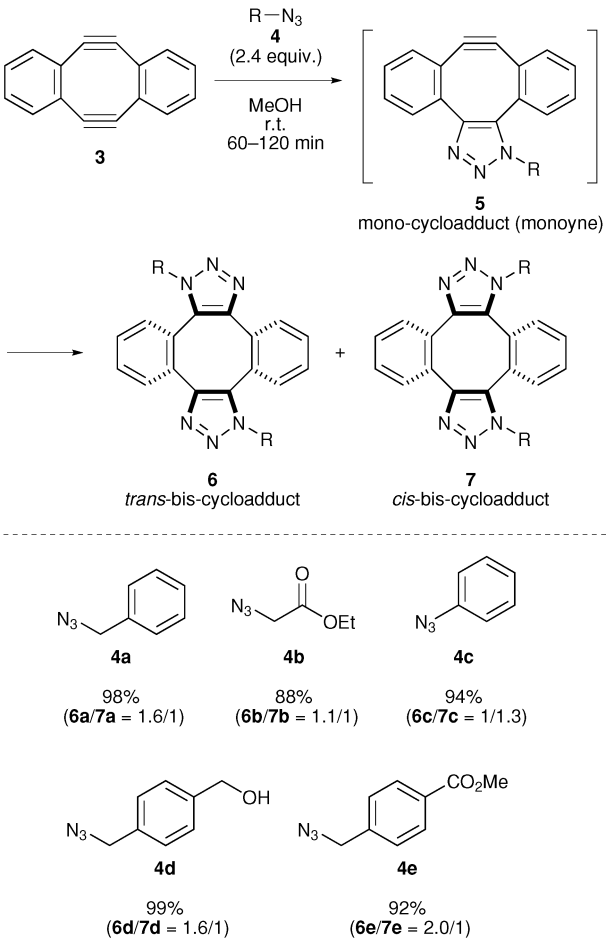
To expand the versatility of the SPAAC reaction, we have conceived the idea of connecting two azides by a bisreactive molecule, thereby avoiding the on-demand preparation of cyclooctyne derivatives. We envisaged that *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**), reported by Sondheimer[10] and now easily available,[11] is an ideal compound because it has two highly strained alkyne bonds[12] ready to react spontaneously.[13] Herein, we show the catalyst-free dual annulation of diyne **3** with two different azido-molecules, the strain-promoted "double-click" (SPDC) reaction (Scheme 1), and demonstrate the chemical modification of an azido-biomolecule with a reporter azido-molecule in both *in vitro* and living cells.



Scheme 1 Chemical modification of azido-biomolecules with small azido-molecules by the strain-promoted double-click (SPDC) reaction.

In an initial study, diyne **3** in methanol (8 mM) was treated with an excess amount of benzyl azide (**4a**, 2.4 equiv.) at room temperature (Scheme 2, R = benzyl). After 70 min, diyne **3** completely reacted to give two regioisomeric bis-cycloadducts **6a** (*trans*) and **7a** (*cis*) in 60% and 38% yields, respectively.[14] X-Ray crystallographic analysis showed their unique saddle-shaped structures (Fig. 1).[15] The mono-cycloadduct **5a**, the presumed monoyne intermediate, was neither isolated nor detected even when an equimolar amount of **4a** was used,[14] indicating that the monoyne intermediate is more reactive toward azides than the starting diyne **3**.

The broad substrate scope in the SPDC reaction was shown from the reactions of diyne **3** with various azides, including ethyl azidoacetate (**4b**), phenyl azide (**4c**), 4-(azidomethyl)benzyl alcohol (**4d**) and methyl 4-(azidomethyl)benzoate (**4e**) (Scheme 2).[14–16] Not surprisingly, the reaction of diyne **3** with an equimolar mixture



Scheme 2 SPDC reaction of diyne **3** with various azides.

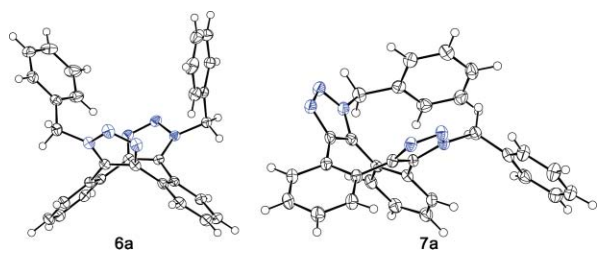


Fig. 1 Structures of bis-cycloadducts **6a** (CCDC 759900) and **7a** (CCDC 759902).

of two different azides gave regioisomeric hetero-cycloadducts as the major products and two pairs of homo-cycloadducts. For example, the reaction of **3** with 1.2 equiv. each of **4d** and **4e** gave regioisomeric heterocycloadducts in 43% combined yield (*trans*/*cis* = 1.2/1), along with homocycloadducts **6d**/**7d** (1.8/1) and **6e**/**7e** (1.2/1) in 28% and 27% yields, respectively.[14]

Fig. 2 shows the computation on the activation barriers and transition states of the SPDC reaction of diyne **3** with methyl azide (**4f**) by a density functional theory (DFT) method.[14,17] The barriers for all cycloadditions were low, confirming the spontaneous reaction of both diyne **3** and monoyne **5f** with **4f** at room temperature. Notably, the activation energy of the second cycloaddition was smaller (+8.8 and +9.5 kcal mol^{-1} for *trans*- and *cis*-adducts, respectively) than that of the first cycloaddition (+12.4

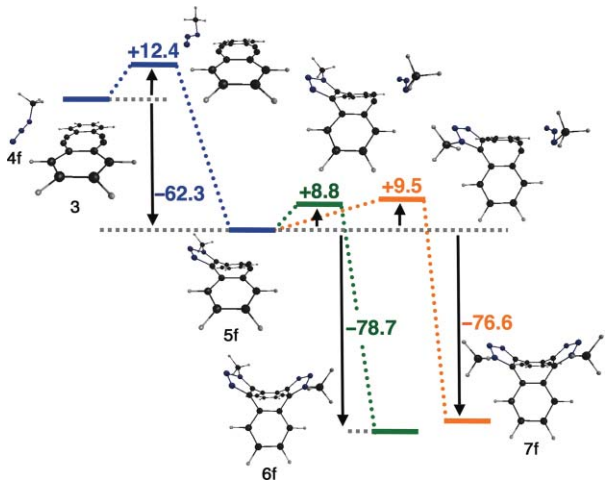


Fig. 2 Potential energy diagram of the SPDC reaction using the B3LYP/6-31G(d) DFT method, showing the relevant stationary points and transition states on the potential energy surfaces for each cycloaddition. All energies (kcal mol^{-1}) include zero-point energy corrections at the level used for geometry optimization.

kcal mol^{-1}), supporting the higher reactivity of the monoyne intermediate.[18] The Kohn–Sham HOMO and LUMO orbital surfaces of each transition states also confirmed the high reactivity of **5f**.[14] The higher reactivity of the monoyne intermediate than the diyne can be attributed to its highly-distorted alkyne bond, which likely arose from the steric repulsion between the substituent on the triazole ring and hydrogen atom on the benzene ring.

To examine the feasibility of chemical modification of azido-biomolecules by the SPDC reaction, we planned to modify an azido-incorporated protein. Among the several methods to prepare azido-proteins,[19,20] we chose to use HaloTag protein that binds covalently with HaloTag ligand possessing a long-chain chloroalkane.[21] The reaction of azido-HaloTag ligand **8** with GST-fused HaloTag protein bound on the GSH-Sepharose resin (HaloTag-GST-resin) gave the desired azido-HaloTag-GST-resin. The fluorescence modification of the azido-HaloTag-GST-resin by the SPDC reaction was attempted by adding diyne **3** into a solution containing azido-HaloTag-GST-resin and TESRA-PEO_3-azide (**9**), an azido-conjugated tetraethylsulforhodamine (TESRA) derivative (Fig. 3(a)). SDS-PAGE analysis showed the fluorescent band (51 kDa) that corresponds to the TESRA-labeled HaloTag-GST protein with nearly 40% of total labeling efficiency (Fig. 3(b), lane 3).[22,23]

Unexpectedly, a sequential procedure, treating azido-HaloTag-GST-resin with **3** and then adding **9** after quick washing out of unreacted **3** by buffer, also achieved the fluorescence labeling of the azido-HaloTag-GST protein with approximately 45% efficiency (Fig. 3(b), lane 7). Under these conditions, the signal for the homo-dimer of the azido-HaloTag-GST protein was substantially undetected.[14] Furthermore, the dimerized product was also hard to detect even in the SPDC modification of soluble azido-HaloTag protein that was free from the resin,[14] indicating that the SPDC reaction between the azido-proteins is not easy to proceed. These results suggest that the monoyne intermediate has a certain lifetime during SPDC reaction under the biological experimental conditions and is available for rapid SPAAC reaction with a different azido-molecule. The noticeable difference between the

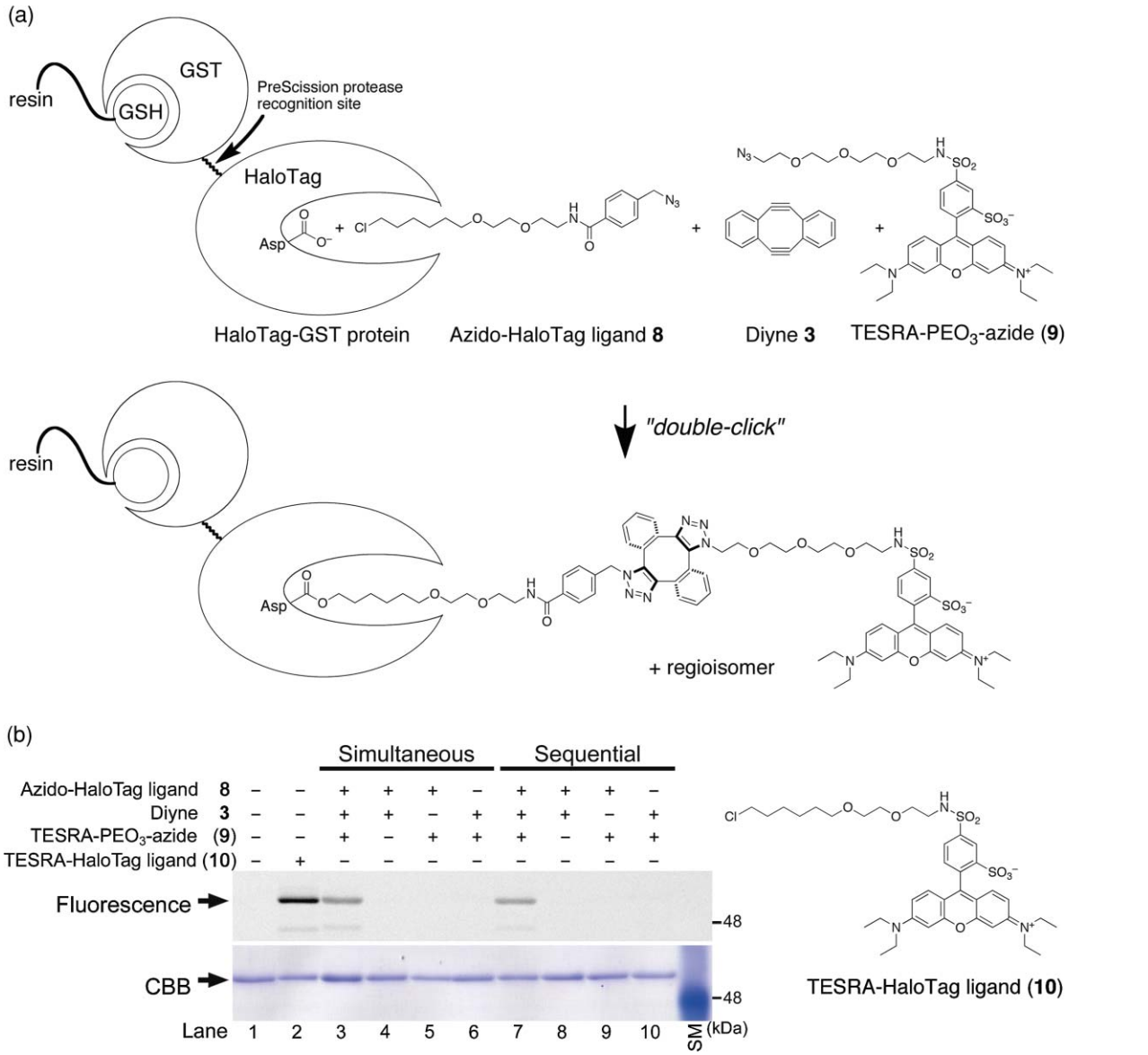


Fig. 3 (a) Schematic view of the SPDC modification of azido-incorporated HaloTag-GST bound on the resin with TESRA-PEO_3-azide (**9**) using diyne **3**. HaloTag protein is a modified haloalkane dehalogenase designed to bind covalently with a specific synthetic ligand, so-called HaloTag ligand bearing a long chloroalkane chain.[21] The chloroalkyl moiety of HaloTag ligand inserts into a binding pocket of HaloTag protein and S_N2 reaction occurs on asparatic acid residue of the protein to form an ester bond. The recognition of the ligands by HaloTag protein is highly specific, ester bond formation occurs rapidly under physiological conditions, and is essentially irreversible. The HaloTag protein was produced in *E. coli* as a glutathione *S*-transferase (GST) tag fusion protein (HaloTag-GST protein), in which a cleavage site recognized by PreScission protease was located between the tags. The HaloTag-GST protein bound on the GSH-Sepharose resin (HaloTag-GST-resin) was incubated with azido-HaloTag ligand **8** to give azido-incorporated HaloTag-GST protein bound on the resin (azido-HaloTag-GST-resin). The azido-HaloTag-GST-resin was incubated with diyne **3** and TESRA-PEO_3-azide (**9**) (simultaneous modification), or with **3** followed by with **9** (sequential modification). The labeled protein was subjected to SDS-PAGE analysis after elution from the GSH-Sepharose resin (b) or to MALDI-TOF-MS analysis after proteolytic excision between GST and HaloTag protein by PreScission protease.[22] (b) SDS-PAGE analysis of GST-fused HaloTag protein eluted from the resin. Purified HaloTag-GST-resin was treated with buffer (–) or azido-HaloTag ligand **8** (100 μM) overnight at 4 °C. The azido-HaloTag-GST-resin was incubated with buffer (–) or diyne **3** (200 μM), and buffer (–) or TESRA-PEO_3-azide (**9**) (200 μM) in the same tube for 15 min at r.t. (Lanes 3–6; simultaneous procedure). The azido-HaloTag-GST-resin was also treated with buffer (–) or **3** for 15 min at r.t., quickly washed with buffer, and then treated with buffer (–) or **9** for 10 min at r.t. (Lanes 7–10; sequential procedure). As a positive control, HaloTag-GST-resin was reacted with TESRA-HaloTag ligand (**10**) (100 μM) overnight at 4 °C (Lane 2). The proteins on the resin were eluted by incubation with SDS-sample buffer containing 10 mM DTT for 5 min at 100 °C, and then subjected to SDS-PAGE. The gel was scanned with a fluorescence image analyzer (Typhoon 8600) and then stained with Coomassie brilliant blue (CBB). SM indicates size marker.

SPDC modification of the azido-protein and the SPDC reaction in an organic solvent, in which the monoyne intermediate could not be detected, can be attributed to the different reaction conditions. The former was performed using ~20 μM of the azido-protein in the presence of excess amounts of diyne **3**, whereas the latter was carried out with ~20 mM of small azido compound

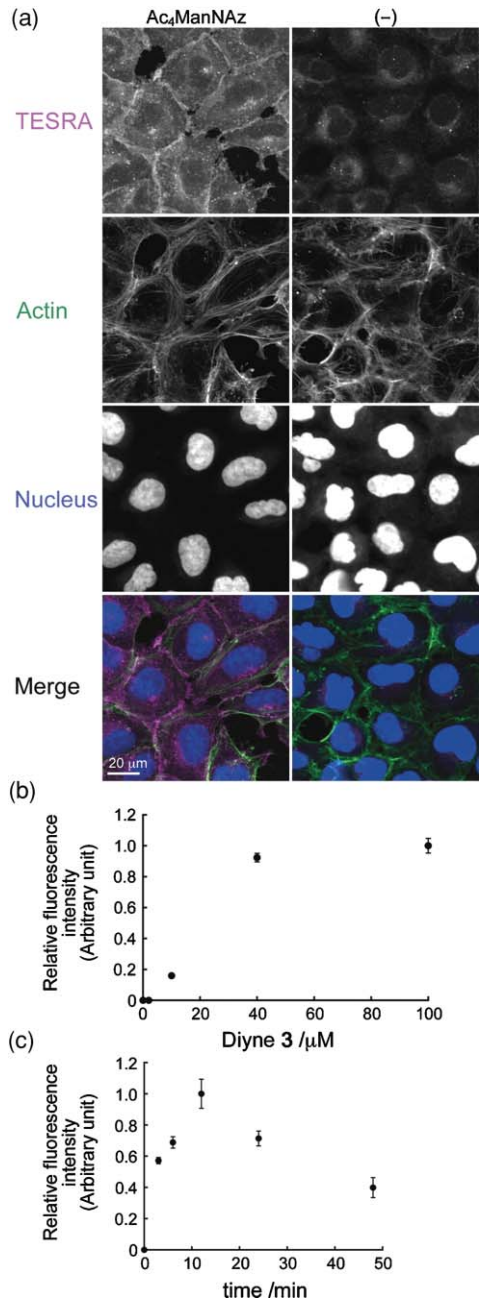


Fig. 4 Cell-surface labeling with diyne **3** and TESRA-PEO_3-azide (**9**). HEK293 cells were incubated for 2 days in the absence (–) or presence of Ac_4ManNAz (100 μM). (a) The azidosugar-incorporated cells were incubated with **3** (40 μM) for 20 min at 37 °C, quickly rinsed with buffer, and then treated with **9** (40 μM) for 20 min at 37 °C. The labeled cells were fixed, and then stained with Alexa Fluor 488-conjugated phalloidin and TO-PRO-3 to visualize cytoskeletal actin fibers and nuclei, respectively. (b) The azidosugar-incorporated cells were incubated with **3** (0 to 100 μM) for 20 min at 37 °C, quickly rinsed, and then treated with **9** (40 μM) for 20 min at 37 °C. Relative fluorescence intensity was determined by densitometric analysis of the fluorescence images. (c) The azidosugar-incorporated cells were incubated with **3** (40 μM) for various times (0 to 48 min) at 37 °C, quickly rinsed, then treated with **9** (40 μM) for 20 min at 37 °C, and analyzed as in (b).

toward 8 mM of diyne **3**. Thus, the dilute condition as well as the lower mobility of the protein would largely reduce the frequency of coming close to each other and prevent the dimerization of azido-proteins.

In order to apply the SPDC modification in living cells, we examined fluorescence labeling of azido-glycoconjugates on the cell surface. HEK293 cells, cultured with a medium containing tetraacetylated *N*-azidoacetyl-D-mannosamine (Ac_4ManNAz),[24] was incubated with diyne **3** and, after quick rinsing with buffer to remove unreacted **3**, TESRA-PEO_3-azide (**9**) was added. Consequently, the azidosugar-incorporated cells exhibited reliable fluorescent signals in the boundary between the cells (Fig. 4(a), left panels), marking a sharp contrast with the control cells which showed negligible signals (Fig. 4(a), right panels). Optimal results were obtained using diyne **3** at the concentrations of 40 to 100 μM (Fig. 4(b)).[25] Maximum labeling efficiency was achieved by treating **3** (40 μM) within 20 min (Fig. 4(c)). Long-time incubation reduced the labeling efficiency (Fig. 4(c)), indicating the time-dependent degradation of the highly reactive monoyne intermediate. The commercially available Alexa Fluor 488 azide was also used in both sequential and simultaneous procedures.[14]

Furthermore, the SPDC modification of azido-glycoconjugates with **3** and a fluorescein-conjugated azide showed a comparable result to that of the single-click modification using a fluorescein derivative of monoyne **2a**.[14] The comparable reactivity of diyne **3** and monoyne **2a** (R = H) was also supported by the competition experiment,[26] as well as kinetic[27] and computational studies.[28]

In conclusion, we have demonstrated the practical utility of the Sondheimer diyne **3** as a bisreactive molecular hinge in modification of azido-biomolecules with a functional small azido-compound. Considering the ready availabilities of diyne **3** and diverse functional azido-compounds, the SPDC reaction provides a facile method to prepare various functional biomolecules.

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Notes and references

1 H. C. Kolb, M. G. Finn and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2001, **40**, 2004–2021.
2 (*a*) C. W. Tornøe, C. Christensen and M. Meldal, *J. Org. Chem.*, 2002, **67**, 3057–3064; (*b*) V. V. Rostovtsev, L. G. Green, V. V. Fokin and K. B. Sharpless, *Angew. Chem., Int. Ed.*, 2002, **41**, 2596–2599.
3 For reviews, see: (*a*) *Click Chemistry for Biotechnology and Materials Science*, ed. J. Lahann, John Wiley & Sons, West Sussex, 2009; (*b*) M. Meldal and C. W. Tornøe, *Chem. Rev.*, 2008, **108**, 2952–3015, and references cited therein.
4 E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974–6998.
5 (*a*) N. J. Agard, J. A. Prescher and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2004, **126**, 15046–15047; (*b*) N. J. Agard, J. M. Baskin, J. A. Prescher, A. Lo and C. R. Bertozzi, *ACS Chem. Biol.*, 2006, **1**, 644–648.
6 G. Wittig and A. Krebs, *Chem. Ber.*, 1961, **94**, 3260–3275.
7 (*a*) J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793–16797; (*b*) J. A. Codelli,

J. M. Baskin, N. J. Agard and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2008, **130**, 11486–11493; (*c*) S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664–667; (*d*) S. T. Laughlin and C. R. Bertozzi, *ACS Chem. Biol.*, 2009, **4**, 1068–1072; (*e*) P. V. Chang, J. A. Prescher, E. M. Sletten, J. M. Baskin, I. A. Miller, N. J. Agard, A. Lo and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 1821–1826; (*f*) J. C. Jewett, E. M. Sletten and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2010, **132**, 3688–3690; (*g*) J. M. Baskin, K. W. Dehnert, S. T. Laughlin, S. L. Amacher and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 10360–10365; For reviews, see: (*h*) C. R. Becer, R. Hoogenboom and U. S. Schubert, *Angew. Chem., Int. Ed.*, 2009, **48**, 4900–4908; (*i*) M. F. Debets, C. W. J. van der Doelen, F. P. J. T. Rutjes and F. L. van Delft, *ChemBioChem*, 2010, **11**, 1168–1184; (*j*) J. C. Jewett and C. R. Bertozzi, *Chem. Soc. Rev.*, 2010, **39**, 1272–1279.

8 (*a*) X. Ning, J. Guo, M. A. Wolfert and G.-J. Boons, *Angew. Chem., Int. Ed.*, 2008, **47**, 2253–2255; (*b*) A. A. Poloukhtine, N. E. Mbua, M. A. Wolfert, G.-J. Boons and V. V. Popik, *J. Am. Chem. Soc.*, 2009, **131**, 15769–15776.

9 M. F. Debets, S. S. van Berkel, S. Schoffelen, F. P. J. T. Rutjes, J. C. M. van Hest and F. L. van Delft, *Chem. Commun.*, 2010, **46**, 97–99.

10 H. N. C. Wong, P. J. Garratt and F. Sondheimer, *J. Am. Chem. Soc.*, 1974, **96**, 5604–5605.

11 (*a*) A. Orita, D. Hasegawa, T. Nakano and J. Otera, *Chem.–Eur. J.*, 2002, **8**, 2000–2004; (*b*) S. Chaffins, M. Brettreich and F. Wudl, *Synthesis*, 2002, 1191–1194.

12 R. Destro, T. Pilati and M. Simonetta, *J. Am. Chem. Soc.*, 1975, **97**, 658–659.

13 (*a*) H. N. C. Wong and F. Sondheimer, *Tetrahedron*, 1981, **37**, 99–109; (*b*) H. Durr, G. Klauck, K. Peters and H. G. von Schnering, *Angew. Chem., Int. Ed. Engl.*, 1983, **22**, 332–333; (*c*) Y.-M. Man, T. C. W. Mak and H. N. C. Wong, *J. Org. Chem.*, 1990, **55**, 3214–3221.

14 See the Supplementary Information (ESI) for the details.

15 The X-ray crystallographic data for compounds **6a** (CCDC 759900), **6b** (CCDC 759901), **6c** (CCDC 759898), **6d** (CCDC 759899), **7a** (CCDC 759902), **7b** (759903), **7c** (CCDC 759905) and **7d** (CCDC 759904) can be obtained free of charge from the Cambridge Crystallographic Data Centre (CCDC) *via* www.ccdc.cam.ac.uk/data_request/cif. See ESI.

16 The geometries of **6e** and **7e** were confirmed by their reduction ($LiAlH_4$/THF) to the corresponding diols **6d** and **7d**, respectively.

17 K. Chenoweth, D. Chenoweth and W. A. Goddard III, *Org. Biomol. Chem.*, 2009, **7**, 5255–5258.

18 Calculations based on distortion/interaction model showed the similar trend. See ESI. For, distortion/interaction model, see F. Schoenebeck, D. H. Ess, G. O. Jones and K. N. Houk, *J. Am. Chem. Soc.*, 2009, **131**, 8121–8133, and references cited therein.

19 (*a*) J. T. Ngo, J. A. Champion, A. Mahdavi, I. C. Tanrikulu, K. E. Beatty, R. E. Connor, T. H. Yoo, D. C. Dieterich, E. M. Schuman and D. A. Tirrell, *Nat. Chem. Biol.*, 2009, **5**, 715–717; (*b*) A. J. de Graaf, M. Kooijman, W. E. Hennink and E. Mastrobattista, *Bioconjugate Chem.*, 2009, **20**, 1281–1295, and references cited therein.

20 (*a*) T. Hosoya, T. Hiramatsu, T. Ikemoto, M. Nakanishi, H. Aoyama, A. Hosoya, T. Iwata, K. Maruyama, M. Endo and M. Suzuki, *Org. Biomol. Chem.*, 2004, **2**, 637–641; (*b*) T. Hosoya, T. Hiramatsu, T. Ikemoto, H. Aoyama, T. Ohmae, M. Endo and M. Suzuki, *Bioorg. Med. Chem. Lett.*, 2005, **15**, 1289–1294; (*c*) T. Ikemoto, T. Hosoya, K. Takata, H. Aoyama, T. Hiramatsu, H. Onoe, M. Suzuki and M. Endo, *Diabetes*, 2009, **58**, 2802–2812; (*d*) R. Kohta, Y. Kotake, T. Hosoya, T. Hiramatsu, Y. Otsubo, H. Koyama, Y. Hirokane, Y. Yokoyama, H. Ikeshoji, K. Oofusa, M. Suzuki and S. Ohta, *J. Neurochem.*, 2010, DOI: 10.1111/j.1471-4159.2010.06576.x.

21 (*a*) Y. Zhang, M.-k. So, A. M. Loening, H. Yao, S. S. Gambhir and J. Rao, *Angew. Chem., Int. Ed.*, 2006, **45**, 4936–4940; (*b*) G. V. Los, L. P. Encell, M. G. McDougall, D. D. Hartzell, N. Karassina, C. Zimprich, M. G. Wood, R. Learish, R. F. Ohana, M. Urh, D. Simpson, J. Mendez, K. Zimmerman, P. Otto, G. Vidugiris, J. Zhu, A. Darzins, D. H. Klaubert, R. F. Bulleit and K. V. Wood, *ACS Chem. Biol.*, 2008, **3**, 373–382.

22 The increase of molecular weight of the protein at each step was clearly confirmed by MALDI-TOF-MS analysis. See ESI.

23 The labeling efficiency was estimated by comparing the fluorescence intensity with that of HaloTag protein labeled directly with TESRA-HaloTag ligand (**10**) (Fig. 3(b), lane 2), in which the labeling mostly proceeded as judged by MALDI-TOF-MS analysis. See ESI.

24 (*a*) E. Saxon and C. R. Bertozzi, *Science*, 2000, **287**, 2007–2010; (*b*) S. T. Laughlin and C. R. Bertozzi, *Nat. Protoc.*, 2007, **2**, 2930–2944; (*c*) S. T. Laughlin and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 12–17.

25 A dose-dependent increase in fluorescence intensity by **9** (0 to 40 μM) was observed. See ESI.

26 Stirring equimolar amounts of diyne **3**, monoyne **2a** (R = H) and benzyl azide (**4a**) in methanol (24 h, r.t.) gave bis-cycloadducts **6a**/**7a** and mono-cycloadducts, coupled products of **2a** and **4a**, in approximately 1 : 1 molar ratio. See ESI.

27 The first cycloaddition is the rate-determining step of the SPDC reaction. The second-order rate constant for the reaction of diyne **3** with benzyl azide (**4a**) was determined to be $(6.29 \pm 0.05) \times 10^{-2}$ M^{-1} s^{-1} (MeOH, 25 °C) by monitoring the time-dependent decrease of absorbance of **3** in the presence of excess amounts of **4a**. It was comparable to that of reported monoyne **2a** (R = H), $(5.67 \pm 0.27) \times 10^{-2}$ M^{-1} s^{-1} (MeOH, 25 °C). See ESI and ref. 8b.

28 The activation energies for cycloaddition of monoyne **2a** (R = H) with methyl azide (**4f**) were within +11.9 to +14.0 kcal mol^{-1}, which were comparable to that for the first cycloaddition of diyne **3** with **4f**. See ESI and ref. 17.