Supplementary Information for:

Strain-Promoted Double-Click Reaction for Chemical Modification of Azido-Biomolecules

Isao Kii,*[a] Akira Shiraishi,[a] Toshiyuki Hiramatsu,[a,b] Takeshi Matsushita,[c] Hidehiro Uekusa,[d] Suguru Yoshida,[b] Makoto Yamamoto,[e] Akira Kudo,[a] Masatoshi Hagiwara[e] and Takamitsu Hosoya*[a,b]

[a]*Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501, Japan.*

[b]*Laboratory of Chemical Biology, Graduate School of Biomedical Science, Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University and SORST, Japan Science and Technology Agency (JST), 2-3-10 Kanda-Surugadai, Chiyoda-ku, Tokyo 101-0062, Japan.*

[c]*Goi Research Center, Chisso Petrochemical Corporation, 5-1 Goikaigan, Ichihara, Chiba 290-8551, Japan.*

[d]*Department of Chemistry and Materials Science, Graduate School of Science and Engineering, Tokyo Institute of Technology, 2-12-1 Ookayama, Meguro-ku, Tokyo 152-8551, Japan.*

[e]*Department of Functional Genomics, Medical Research Institute, Graduate School of Biomedical Science, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-851, Japan.*

*Correspondence: ikii@bio.titech.ac.jp (IK), thosoya.cb@tmd.ac.jp (TH).

SS. Chemical experiments.

SS1. General remarks.

sym-Dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**),[S1] phenyl azide (**4c**),[S2] 4-(azidomethyl)benzyl alcohol (**4d**),[S3] methyl 4-(azidomethyl)benzoate (**4e**),[S4] *tert*-butyl *N*-[2-{2-(6-chlorohexyloxy)ethoxy}ethyl]carbamate (**11**),[S5] succinimido 4-(azidomethyl)benzoate (**13**),[S6] lissamine rhodamine B sulfonyl chloride (**15**),[S7] 2-azidoethylamine (**16**) (~50%, ^{1}H NMR),[S8] 11,12-didehydro-5,6-dihydrodibenzo[*a,e*]cycloocten-5-yl 4-nitrophenyl carbonate (**20**)[S9] and 11,12-didehydro-5,6-dihydrodibenzo[*a,e*]cycloocten-5-ol (**2a**, R = H)[S9] were prepared according to the reported methods.

Benzyl azide (**4a**) (Cat. No. 327-79632), ethyl azidoacetate (**4b**) (Cat. No. 328-44311), trifluoroacetic acid (Cat. No. 208-02741), triethylamine (Cat. No. 202-02646), lithium aluminium hydride (Cat. No. 128-01092), ethylenediamine (Cat. No. 059-00933) and methanol for spectrochemical analysis (Cat. No. 139-13995) were purchased from Wako Pure Chemical Industries Ltd. 11-Azido-3,6,9-trioxaundecan-1-amine (**14**) (≥90%, GC) (Cat. No. 17758) and fluorescein isothiocyanate isomer I (5-isothiocyanatofluorescein, FITC) (**17**) (≥90%, HPLC) (Cat. No. F7250) were purchased from Sigma-Aldrich Japan K.K. Dess–Martin periodinane (Cat. No. D2045) was purchased from Tokyo Chemical Industry Co., Ltd. All other chemical reagents used were commercial grade and used as received.

Analytical thin-layer chromatography (TLC) was performed on precoated (0.25 mm) silica-gel plates (Merck Chemicals, Silica Gel 60 F_{254}, Cat. No. 1.05715). Column chromatography was conducted using silica-gel (Kanto Chemical Co., Inc., Silica Gel 60N, spherical neutral, particle size 40–50 μm, Cat. No. 37563-85 or particle size 63–210 μm, Cat. No. 37565-85).

Melting points (Mp) were measured on a YANACO MP-J3 instrument and are uncorrected.

^{1}H and ^{13}C NMR spectra were obtained with a Varian Mercury 300 spectrometer at 300 and 75.5 MHz, respectively. $CDCl_3$ (CIL, Cat. No. DLM-7TB), DMSO-d_6 (CIL, Cat. No. DLM-10) and CD_3OD (CIL, Cat. No. DLM-24-10) were used as solvents for obtaining NMR spectra. Chemical shifts (δ) are given in parts per million (ppm) downfield from $(CH_3)_4Si$ (δ 0.00 for ^{1}H NMR in $CDCl_3$) or the solvent peak (δ 2.49 for ^{1}H NMR and δ 39.5 for ^{13}C NMR in DMSO-d_6, δ 3.30 for ^{1}H NMR in CD_3OD and δ 77.0 for ^{13}C NMR in $CDCl_3$) as an internal reference with coupling constants (*J*) in hertz (Hz). The abbreviations s, d, t, q, m and br signify singlet, doublet, triplet, quartet, multiplet and broad, respectively.

IR spectra were measured by diffuse reflectance method on a Shimadzu IRPrestige-21 spectrometer attached with DRS-8000A with the absorption band given in cm^{-1}.

The absorbance spectra (UV) were measured with a Shimadzu UV-3100 spectrophotometer at 25 °C using a quartz cuvette (10 mm light path).

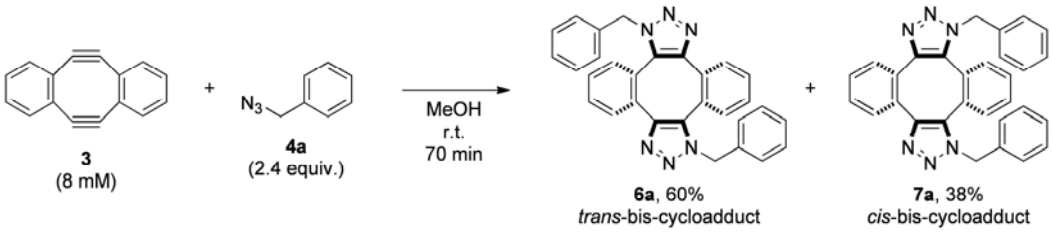
The fluorescence spectra (FL) were measured with a Shimadzu RF-5300PC spectrofluorophotometer (emission and excitation bandwidth, 3 nm; response, 0.5 sec; scan speed, medium) at 25 °C using a quartz cuvette (10 mm light path).

High-resolution mass spectra (HRMS) were measured on a Bruker micrOTOF mass spectrometer under positive electrospray ionization (ESI^+) conditions at Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University, or a JEOL JMS-700 mass spectrometer under electron impact ionization (EI) conditions or positive fast atom bombardment (FAB^+) conditions at the Center for Advanced Materials Analysis (Suzukakedai), Technical Department, Tokyo Institute of Technology.

Intensity data of X-ray crystallographical analyses were collected on a Rigaku R-AXIS RAPID diffractometer. The structures were solved by direct methods and refined by the full-matrix least-squares on F^2 (SHELXL97). These data can be obtained free of charge from the Cambridge Crystallographic Data Centre (CCDC) via www.ccdc.cam.ac.uk/data_request/cif.

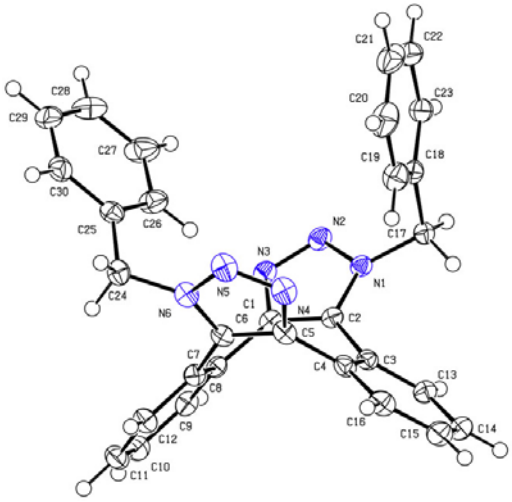
SS2. SPDC reaction of diyne **3** with various azides.

SS2-1. SPDC reaction of diyne **3** with benzyl azide (**4a**).



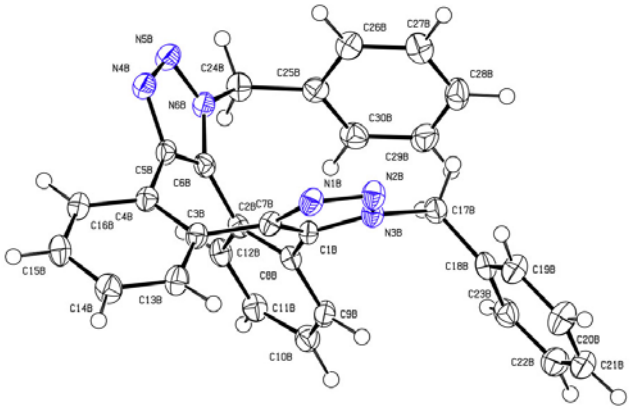
To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of benzyl azide (**4a**) (63.9 mg, 480 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 70 min at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2 only to CH_2Cl_2/MeOH = 6/1) to give *trans*-bis-cycloadduct **6a** (55.8 mg, 120 μmol, 59.9%) and *cis*-bis-cycloadduct **7a** (35.2 mg, 75.4 μmol, 37.8%). The geometries of these compounds were confirmed by X-ray crystallographical analyses (CCDC 759900 (**6a**) and CCDC 759902 (**7a**)).

1,8-Dibenzyl-1,8-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**6a**)



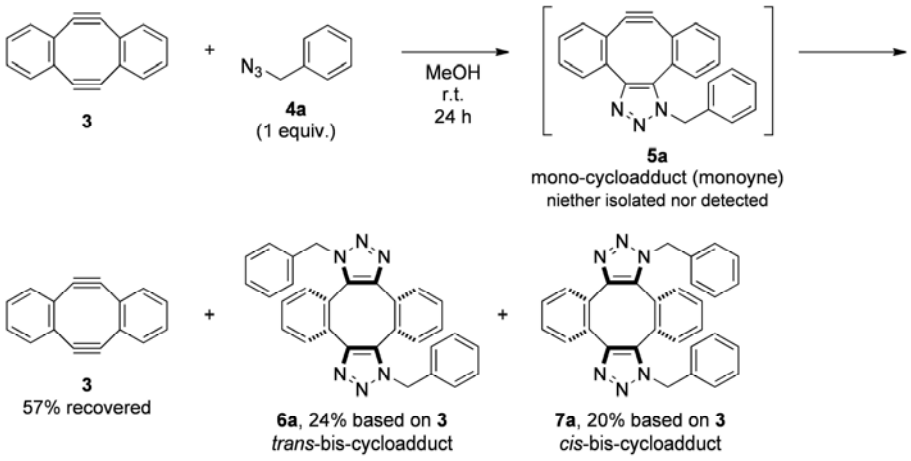
Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 230–232 °C; R_f = 0.26 (*n*-hexane/EtOAc = 1/1); R_f = 0.69 (CH_2Cl_2/MeOH = 9/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.31 (d, 2H, *J* = 15.3 Hz), 5.50 (d, 2H, *J* = 15.3 Hz), 6.94–7.01 (m, 4H), 7.09 (dd, 2H, *J* = 0.8, 7.6 Hz), 7.22–7.30 (m, 6H), 7.40 (ddd, 2H, *J* = 1.3, 7.6, 7.6 Hz), 7.52 (ddd, 2H, *J* = 1.3, 7.6, 7.6 Hz), 7. 71 (dd, 2H, *J* = 0.8, 7.6 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 52.1 (2C), 126.4 (2C), 127.0 (4C), 128.2 (2C), 128.7 (2C), 128.8 (4C), 129.9 (2C), 130.2 (2C), 131.2 (2C), 132.6 (2C), 134.9 (2C), 135.1 (2C), 145.0 (2C); IR (KBr, cm^{-1}) 706, 731,764, 910, 984, 1028, 1215, 1250, 1350, 1454, 1497, 1514, 3063; HRMS (ESI^+) *m*/z 467.1984 ($[M+H]^+$, $C_{30}H_{23}N_6^+$ requires 467.1979).

1,10-Dibenzyl-1,10-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d*']bis([1,2,3]triazole) (**7a**)



Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 274–277 °C; R_f = 0.26 (*n*-hexane/EtOAc = 1/1); R_f = 0.44 (CH_2Cl_2/MeOH = 9/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 4.89 (d, 2H, *J* = 15.5 Hz), 5.31 (d, 2H, *J* = 15.5 Hz), 6.94–7.04 (m, 4H), 7.04–7.14 (m, 2H), 7.24–7.36 (m, 6H), 7.36–7.45 (m, 2H), 7.45–7.54 (m, 2H), 7.62–7.72 (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 52.0 (2C), 127.2 (4C), 127.9 (2C), 128.3 (2C), 128.8 (4C), 129.1 (2C), 129.8 (2C), 130.3 (2C), 130.7 (2C), 130.9 (2C), 133.6 (2C), 135.3 (2C), 146.2 (2C); IR (KBr, cm^{-1}) 698, 729, 766, 910, 984, 1028, 1211, 1246, 1344, 1427, 1454, 1497, 1516, 3061; HRMS (ESI^+) *m*/z 467.1982 ($[M+H]^+$, $C_{30}H_{23}N_6^+$ requires 467.1979).

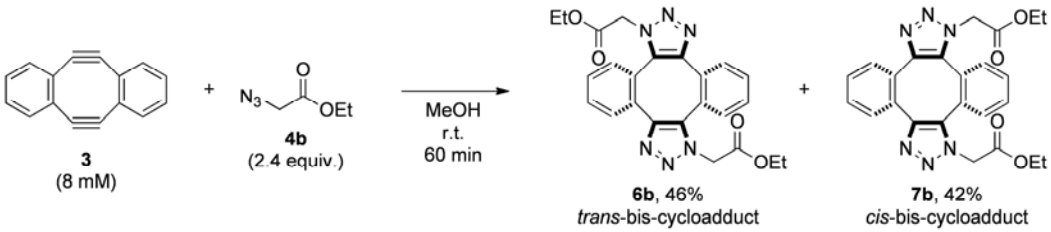
SS2-2. SPDC reaction of diyne **3** with an equimolar amount of benzyl azide (**4a**).



To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of benzyl azide (**4a**) (26.6 mg, 200 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 24 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2 only to CH_2Cl_2/MeOH = 6/1) to give *trans*-bis-cycloadduct **6a** (22.0 mg, 47.2 μmol, 23.6% based on azide **4a**) and *cis*-bis-cycloadduct **7a** (19.0 mg, 40.7 μmol, 20.4% based on diyne **3**) along with recovery of starting diyne **3** (22.7 mg, 113 μmol, 56.8% recovered).

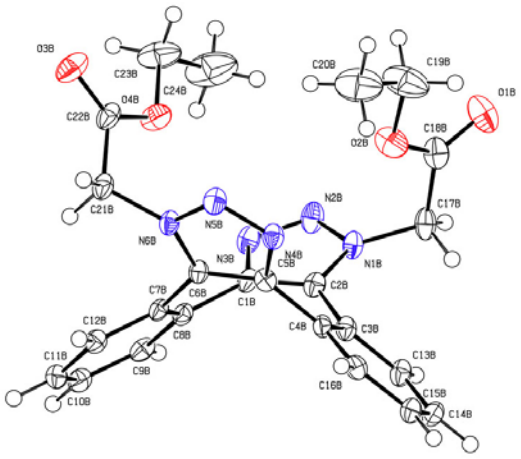
The combined yield of bis-cycloadducts **6a**/**7a** and recovered diyne **3** was quantitative, indicating that the monoyne intermediate could not isolated under the reaction conditions examined.

SS2-3. SPDC reaction of diyne **3** with ethyl azidoacetate (**4b**).



To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of ethyl azidoacetate (**4b**) (55.0 μL, 480 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 60 min at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 1/1) to give *trans*-bis-cycloadduct **6b** (41.9 mg, 91.5 μmol, 45.8%) and *cis*-bis-cycloadduct **7b** (38.8 mg, 84.7 μmol, 42.4%). The geometries of these compounds were confirmed by X-ray crystallographical analyses (CCDC 759901 (**6b**) and CCDC 759903 (**7b**)).

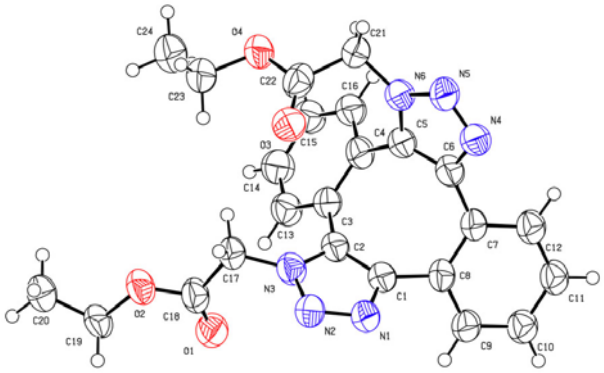
1,8-Di(ethoxycarbonylmethyl)-1,8-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d*']bis([1,2,3]triazole) (**6b**)



Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 204–205 °C; R_f = 0.55 (*n*-hexane/EtOAc = 1/3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.23 (t, 6H, *J* = 7.1 Hz), 4.10–4.34 (m, 4H), 4.87 (d, 2H, *J* = 17.4 Hz), 5.09 (d, 2H, *J* = 17.4 Hz), 7.31 (d, 2H, *J* = 7.1 Hz), 7.48 (dd, 2H, *J* = 7.1,

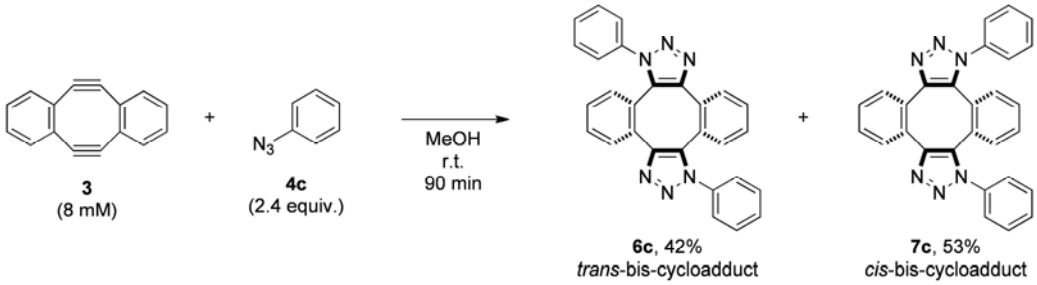
7.1 Hz), 7.57 (dd, 2H, *J* = 7.1, 7.1 Hz), 7.78 (d, 2H, *J* = 7.1 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.8 (2C), 49.4 (2C), 62.3 (2C), 125.7 (2C), 129.0 (2C), 129.1 (2C), 130.3 (2C), 131.5 (2C), 132.4 (2C), 135.3 (2C), 144.4 (2C), 166.3 (2C); IR (KBr, cm^{-1}) 737, 766, 779, 876, 910, 986, 1022, 1134, 1161, 1211, 1256, 1348, 1364, 1418, 1474, 1516, 1748, 2982; HRMS (ESI^+) *m*/*z* 459.1788 ($[M+H]^+$, $C_{24}H_{23}N_6O_4^+$ requires 459.1775).

1,10-Di(ethoxycarbonylmethyl)-1,10-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d*']bis([1,2,3]triazole) (**7b**)



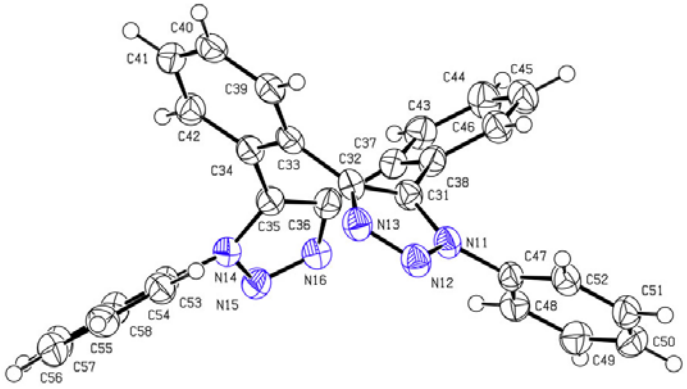
Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 214–217 °C; R_f = 0.38 (*n*-hexane/EtOAc = 1/3); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.26 (t, 6H, *J* = 7.1 Hz), 4.21 (q, 4H, *J* = 7.1 Hz), 5.03 (d, 2H, *J* = 17.4 Hz), 5.13 (d, 2H, *J* = 17.4 Hz), 7.35–7.44 (m, 2H), 7.47–7.59 (m, 4H), 7.66–7.76 (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 13.9 (2C), 49.3 (2C), 62.2 (2C), 128.3 (2C), 129.0 (2C), 129.8 (2C), 130.19 (2C), 130.22 (2C), 130.8 (2C), 134.5 (2C), 145.6 (2C), 166.5 (2C); IR (KBr, cm^{-1}) 733, 768, 799, 876, 912, 986, 1020, 1132, 1161, 1211, 1250, 1346, 1362, 1414, 1474, 1518, 1748, 2984; HRMS (ESI^+) *m*/*z* 459.1783 ($[M+H]^+$, $C_{24}H_{23}N_6O_4^+$ requires 459.1775).

SS2-4. SPDC reaction of diyne **3** with phenyl azide (**4c**).



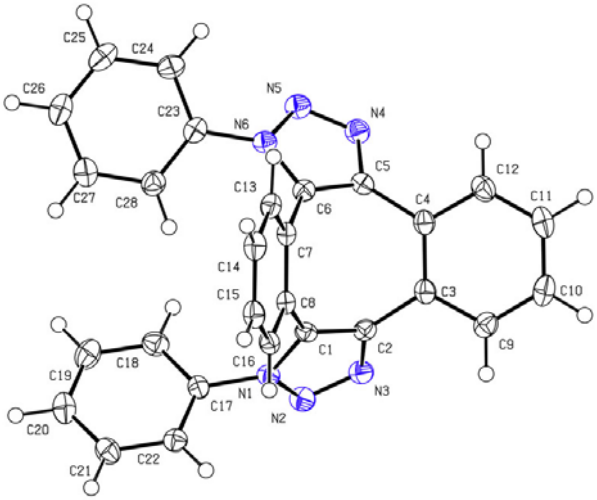
To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of phenyl azide (**4c**) (57.2 mg, 480 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 90 min at the same temperature, the mixture was concentrated under reduced pressure. The crude product was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 4/1) to give *trans*-bis-cycloadduct **6c** (36.5 mg, 83.2 μmol, 41.7%) and *cis*-bis-cycloadduct **7c** (46.1 mg, 105 μmol, 52.6%). The geometries of these compounds were confirmed by X-ray crystallographical analyses (CCDC 759898 (**6c**) and CCDC 759905 (**7c**)).

1,8-Dihydro-1,8-diphenyldibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d*′]bis([1,2,3]triazole) (**6c**)



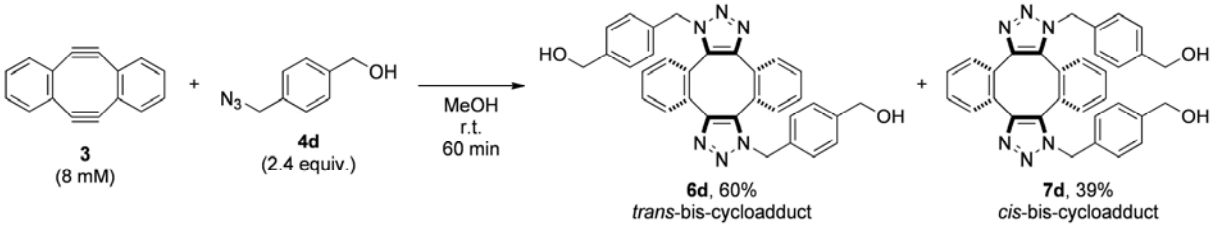
Recrystallized from *n*-hexane/CH_2Cl_2 to give colorless crystals; Mp >300 °C; R_f = 0.23 (*n*-hexane/EtOAc = 3/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.84 (dd, 2H, *J* = 1.2, 7.8 Hz), 7.21 (ddd, 2H, *J* = 1.2, 7.8, 7.8 Hz), 7.29–7.43 (m, 10H), 7.48 (ddd, 2H, *J* = 1.2, 7.8, 7.8 Hz), 7.83 (dd, 2H, *J* = 1.2, 7.8 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 125.0 (4C), 126.7 (2C), 128.8 (2C), 129.1 (2C), 129.3 (4C), 130.0 (2C), 130.8 (2C), 131.3 (2C), 131.9 (2C), 134.2 (2C), 135.9 (2C), 145.8 (2C); IR (KBr, cm^{-1}) 692, 734, 768, 997, 1265, 1361, 1497, 1512, 1595; HRMS (ESI^+) *m*/z 439.1667 ($[M+H]^+$, $C_{28}H_{19}N_6^+$ requires 439.1666).

1,10-Dihydro-1,10-diphenyldibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d*′]bis([1,2,3]triazole) (**7c**)



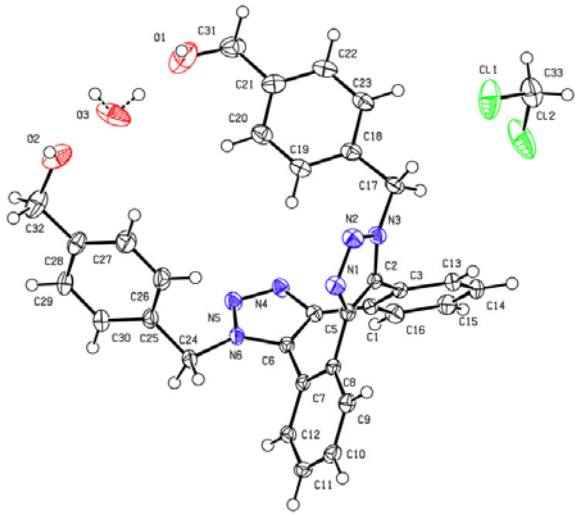
Recrystallized from *n*-hexane/CH_2Cl_2 to give colorless crystals; Mp >300 °C; R_f = 0.13 (*n*-hexane/EtOAc = 3/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.87–6.95 (m, 2H), 7.12–7.21 (m, 2H), 7.39–7.51 (m, 10H), 7.52–7.62 (m, 2H), 7.77–7.85 (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 126.1 (4C), 127.4 (2C), 129.4 (4C), 129.6 (2C), 129.8 (2C), 130.3 (2C), 130.6 (2C), 131.4 (2C), 131.6 (2C), 134.1 (2C), 135.8 (2C), 145.1 (2C); IR (KBr, cm^{-1}) 527, 608, 687, 734, 762, 997, 1069, 1132, 1175, 1263, 1358, 1427, 1476, 1497, 1514, 1595; HRMS (ESI^+) *m*/z 439.1670 ($[M+H]^+$, $C_{28}H_{19}N_6^+$ requires 439.1666).

SS2-5. SPDC reaction of diyne **3** with 4-(azidomethyl)benzyl alcohol (**4d**).



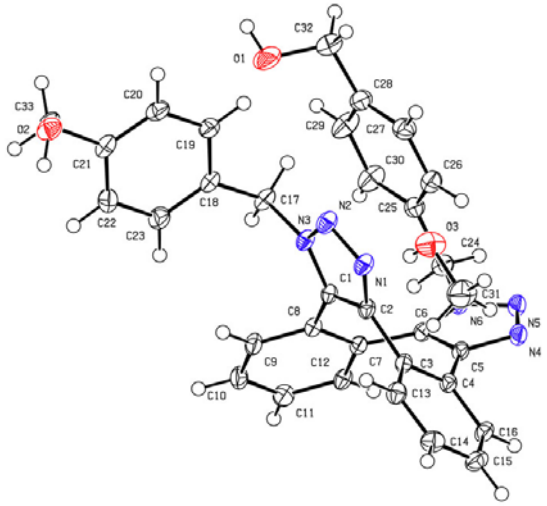
To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of 4-(azidomethyl)benzyl alcohol (**4d**) (78.3 mg, 480 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 60 min at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2/MeOH = 29/1) to give *trans*-bis-cycloadduct **6d** (63.5 mg, 121 μmol, 60.3%) and *cis*-bis-cycloadduct **7d** (40.9 mg, 77.7 μmol, 38.9%). The geometries of these compounds were confirmed by X-ray crystallographical analyses (CCDC 759899 (**6d**) and CCDC 759904 (**7d**)).

1,8-Di[4-(hydroxylmethyl)benzyl]-1,8-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**6d**)



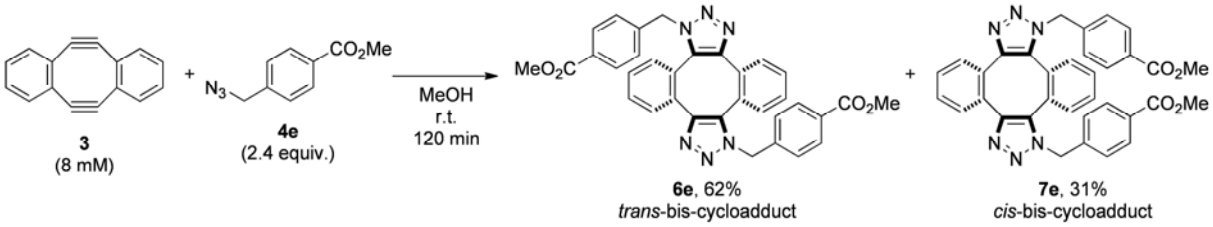
Recrystallized from CH_2Cl_2/MeOH to give colorless crystals; Mp >300 °C (dec.); R_f = 0.45 (*n*-hexane/EtOAc = 1/9); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.38 (br s, 2H), 4.52–4.70 (m, 4H), 5.27 (d, 2H, *J* = 14.7 Hz), 5.29 (d, 2H, *J* = 14.7 Hz), 6.52–6.62 (AA'BB'×2, 4H), 7.06–7.13 (AA'BB'×2, 4H), 7.13–7.20 (m, 2H), 7.38–7.57 (m, 6H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 51.0 (2C), 62.4 (2C), 126.2 (4C), 126.5 (4C), 129.3 (2C), 130.1 (2C), 130.3 (2C), 130.9 (2C), 132.1 (2C), 134.0 (2C), 134.5 (2C), 142.1 (2C), 144.5 (2C), 147.7 (2C); IR (KBr, cm^{-1}) 519, 594, 775, 986, 1028, 1105, 1132, 1215, 1252, 1314, 1352, 1422, 1474, 1514, 1616, 2097, 2868, 3366; HRMS (ESI^+) *m*/*z* 527.2191 ($[M+H]^+$, $C_{32}H_{27}N_6O_2^+$ requires 527.2190).

1,10-Di[4-(hydroxylmethyl)benzyl]-1,10-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**7d**)



Recrystallized from CH_2Cl_2/MeOH to give colorless crystals; Mp >300 °C (dec.); R_f = 0.39 (*n*-hexane/EtOAc = 1/9); ^{1}H NMR (300 MHz, $CDCl_3$) δ 2.03 (t, 2H, *J* = 5.2 Hz), 4.66 (d, 4H, *J* = 5.2 Hz), 4.96 (d, 2H, *J* = 15.7 Hz), 5.33 (d, 2H, *J* = 15.7 Hz), 6.90–7.00 (AA'BB'×2, 4H), 7.08–7.16 (m, 2H) 7.20–7.32 (AA'BB'×2, 4H), 7.40–7.52 (m, 4H), 7.62–7.70 (m, 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 51.3 (2C), 62.5 (2C), 126.8 (4C), 127.0 (2C), 127.1 (4C), 129.2 (2C), 130.2 (2C), 130.3 (2C), 130.7 (2C), 130.8 (2C), 133.5 (2C), 133.8 (2C), 142.5 (2C), 145.0 (2C); IR (KBr, cm^{-1}), 704, 737, 764, 986, 1028, 1134, 1209, 1248, 1265, 1287, 1315, 1346, 1422, 1514, 1634, 2089, 3360; HRMS (ESI$^+$) *m*/z 527.2205 ($[M+H]^+$, $C_{32}H_{27}N_6O_2^+$ requires 527.2190).

SS2-6. SPDC reaction of diyne **3** with methyl 4-(azidomethyl)benzoate (**4e**).



To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (23.5 mL) was added a solution of methyl 4-(azidomethyl)benzoate (**4e**) (91.8 mg, 480 μmol) in MeOH (1.5 mL) at room temperature. After stirring for 120 min at the same temperature, the mixture was concentrated under reduced pressure. The residual solid was placed on a funnel and washed with EtOAc to give pure *cis*-bis-cycloadduct **7e** (36.1 mg, 61.9 μmol, 30.9%). The filtrate was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 1/2 to EtOAc only) to give *trans*-bis-cycloadduct **6e** (71.8 mg, 123 μmol, 61.5%). The geometries of compounds **6e** and **7e** were confirmed by their reduction to corresponding diols **6d** and **7d**, respectively, using $LiAlH_4$ in THF (0 °C to reflux, 7.5 h).

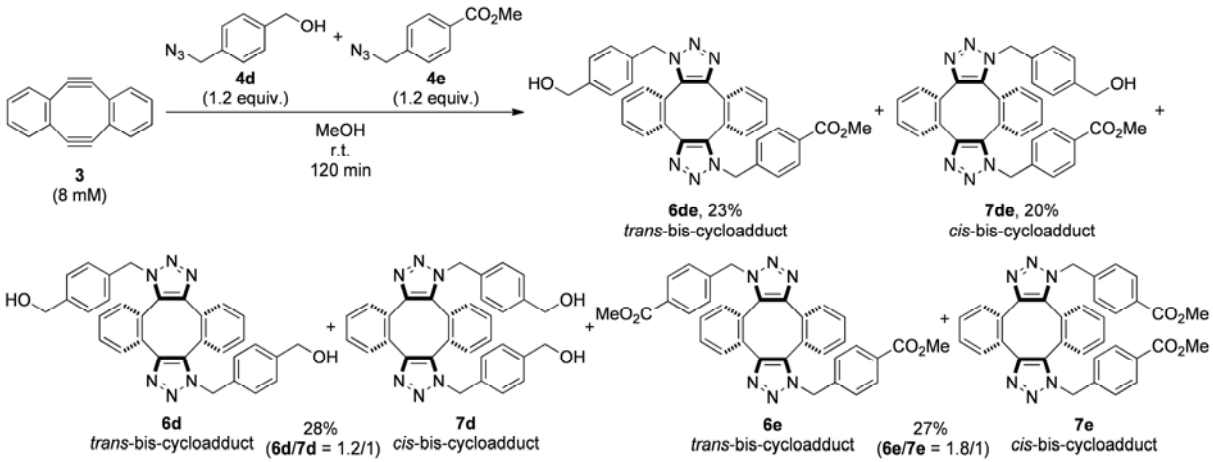
1,8-Di[4-(methoxycarbonyl)benzyl]-1,8-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**6e**)

Colorless solid; Mp 102–104 °C; R_f = 0.31 (*n*-hexane/EtOAc = 2/1);
R_f = 0.27 (CH_2Cl_2/MeOH = 15/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.90 (s, 6H), 5.34 (d, 2H, *J* = 15.9 Hz), 5.57 (d, 2H, *J* = 15.9 Hz), 7.03 (d, 2H, *J* = 7.6 Hz), 7.05-7.12 (AA'BB'×2, 4H), 7.39 (dd, 2H, *J* = 7.6, 7.6 Hz), 7.54 (dd, 2H, *J* = 7.6, 7.6 Hz), 7.73 (d, 2H, *J* = 7.6 Hz), 7.91-8.00 (AA'BB'×2, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 51.0 (2C), 52.1 (2C), 126.0 (2C), 126.5 (4C), 128.9 (2C), 129.3 (4C), 129.4 (2C), 130.1 (2C), 130.4 (2C), 130.7 (2C), 131.8 (2C), 134.6 (2C), 140.9 (2C), 144.8 (2C), 165.6 (2C); IR (KBr, cm^{-1}) 581, 735, 746, 764, 806, 910, 1020, 1111, 1180, 1283, 1352, 1435, 1516, 1614, 1719, 2951; HRMS (ESI^+) *m*/z 583.2104 ($[M+H]^+$, $C_{34}H_{27}N_6O_4^+$ requires 583.2088).

1,10-Di[4-(methoxycarbonyl)benzyl]-1,10-dihydrodibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**7e**)

Colorless solid; Mp 287–290 °C; R_f = 0.31 (*n*-hexane/EtOAc = 2/1);
R_f = 0.27 (CH_2Cl_2/MeOH = 15/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.91 (s, 6H), 4.98 (d, 2H, *J* = 15.9 Hz), 5.36 (d, 2H, *J* = 15.9 Hz), 6.98–7.11 (m, 6H), 7.38–7.45 (m, 2H), 7.46–7.56 (m, 2H), 7.64–7.72 (m, 2H), 7.94–8.00 (AA'BB'×2, 4H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 51.7 (2C), 52.4 (2C), 127.1 (4C), 127.8 (2C), 129.3 (2C), 130.06 (2C), 130.14 (2C), 130.2 (4C), 130.3 (2C), 130.4 (2C), 130.6 (2C), 133.5 (2C), 140.1 (2C), 146.3 (2C), 166.3 (2C); IR (KBr, cm^{-1}) 582, 735, 750, 764, 804, 910, 1020, 1111, 1180, 1283, 1435, 1516, 1614, 1719, 2951; HRMS (ESI^+) *m*/z 583.2092 ($[M+H]^+$, $C_{34}H_{27}N_6O_4^+$ requires 583.2088).

SS2-7. SPDC reaction of diyne **3** with an equimolar mixture of 4-(azidomethyl)benzyl alcohol (**4d**) and methyl 4-(azidomethyl)benzoate (**4e**).



To a solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (40.0 mg, 200 μmol) in MeOH (22 mL) was added a mixture of 4-(azidomethyl)benzyl alcohol (**4d**) (39.2 mg, 240 μmol) and methyl 4-(azidomethyl)benzoate (**4e**) (45.9 mg, 240 μmol) in MeOH (3 mL) at room temperature. After stirring for 120 min at the same temperature, the mixture was concentrated under reduced pressure.

The residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 1/1 to 1/4, then EtOAc only) to give unsymmetrical *trans*-bis-cycloadduct **6de** (21.8 mg, 39.3 μmol, 19.7%) and *cis*-bis-cycloadduct **7de** (25.3 mg, 45.6 μmol, 22.8%), along with regioisomeric mixtures of symmetrical bis-cycloadducts **6d**/**7d** (29.2 mg, 55.5 μmol, 27.8%, **6d**/**7d** = 1.8/1) and **6e**/**7e** (31.4 mg, 53.9 μmol, 27.0%, **6e**/**7e** = 1.2/1). The ratios of regioisomers **6d**/**7d** and **6e**/**7e** were determined by comparing the integration values of benzylic protons on ^{1}H NMR spectrum. The geometries of compounds **6de** and **7de** were confirmed by their reduction to corresponding diols **6d** and **7d**, respectively, using $LiAlH_4$ in THF (0 °C to reflux, 7.5 h).

1,8-Dihydro-1-[4-(hydroxylmethyl)benzyl]-8-[4-(methoxycarbonyl)benzyl]dibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**6de**)

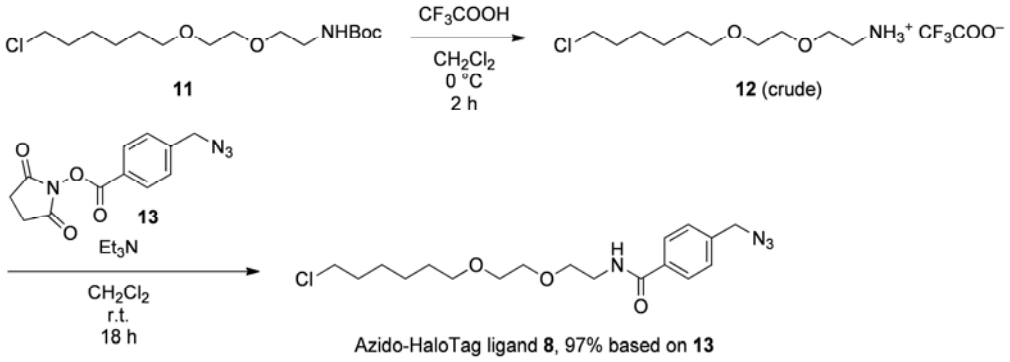
Colorless solid; Mp 116–118 °C; R_f = 0.30 (*n*-hexane/EtOAc = 1/4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.16 (br s, 1H), 3.91 (s, 3H), 4.60 (s, 2H), 5.28–5.40 (m, 2H), 5.51 (d, 1H, *J* = 15.5 Hz), 5.61 (d, 1H, *J* = 15.5 Hz), 6.64–6.72 (AA'BB', 2H), 6.92–7.03 (m, 3H), 7.05–7.12 (AA'BB', 2H), 7.23 (d, 1H, *J* = 7.3 Hz), 7.38 (dd, 1H, *J* = 7.3, 7.3 Hz), 7.42–7.62 (m, 4H), 7.71 (d, 1H, *J* = 7.3 Hz), 7.90–7.98 (AA'BB', 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ51.6, 52.1, 52.6, 64.8, 126.0, 126.5, 126.6 (2C), 127.2 (2C), 127.8 (2C), 128.8, 128.9, 129.67, 129.71, 130.0, 130.1 (3C), 130.4, 131.0, 131.2, 131.9, 132.5, 133.7, 134.7, 134.9, 139.9, 141.3, 144.6, 145.2, 166.4; IR (KBr, cm^{-1}) 583, 748, 961, 986, 1018, 1049, 1109, 1182, 1215, 1281, 1352, 1435, 1514, 1614, 1719, 2068, 2359, 3389; HRMS (ESI^+) *m*/z 555.2150 ($[M+H]^+$, $C_{33}H_{27}N_6O_3^+$ requires 555.2139).

1,10-Dihydro-1-[4-(hydroxylmethyl)benzyl]-10-[4-(methoxycarbonyl)benzyl]dibenzo[3,4:7,8]cycloocta[1,2-*d*:5,6-*d'*]bis([1,2,3]triazole) (**7de**)

Colorless solid; Mp 134–136 °C; R_f = 0.38 (*n*-hexane/EtOAc = 1/4); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.90 (t, 1H, *J* = 5.4 Hz), 3.90 (s, 3H), 4.68 (d, 2H, *J* = 5.4 Hz), 4.89 (d, 1H, *J* = 16.0 Hz), 5.04 (d, 1H, *J* = 16.0 Hz), 5.33 (d, 1H, *J* = 16.0 Hz), 5.39 (d, 1H, *J* = 16.0 Hz), 6.88–6.96 (AA'BB', 2H), 7.00 (d, 1H, *J* = 7.6 Hz), 7.04–7.10 (AA'BB', 2H), 7.15 (d, 1H, *J* = 7.6 Hz), 7.24–7.32 (AA'BB', 2H), 7.34–7.54 (m, 4H), 7.62–7.70 (m, 2H), 7.92–7.99 (AA'BB', 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 51.6, 51.9, 52.3, 64.3, 127.0 (2C), 127.1 (2C), 127.4 (2C), 127.6, 128.0, 129.18, 129.22, 129.9, 130.06, 130.09 (3C), 130.3, 130.5, 130.61, 130.64, 130.7, 133.4, 133.7, 134.1, 140.4, 141.3, 146.1, 146.2, 150.8, 166.4; IR (KBr, cm^{-1}) 471, 579, 750, 984, 1018, 1111, 1209, 1281, 1346, 1416, 1514, 1614, 1715, 2949, 3061, 3416; HRMS (ESI^+) *m*/z 555.2127 ($[M+H]^+$, $C_{33}H_{27}N_6O_3^+$ requires 555.2139).

SS3. Syntheses of HaloTag ligands and fluorescent probes.

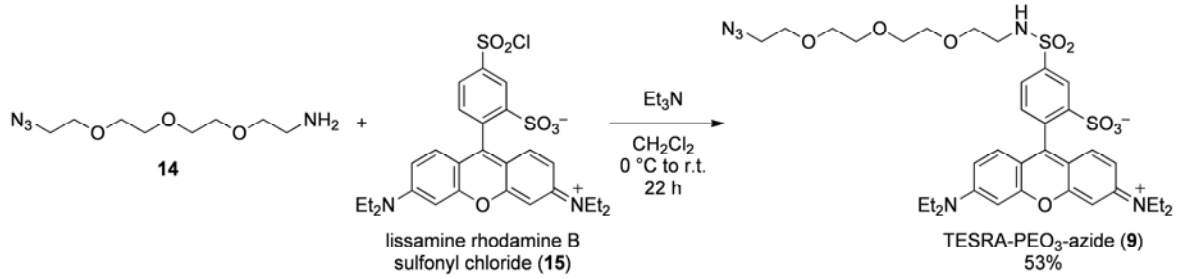
SS3-1. Synthesis of azido-HaloTag ligand **8**.



Under argon atmosphere, to a solution of *tert*-butyl *N*-[2-{2-(6-chlorohexyloxy)ethoxy}ethyl]carbamate (**11**) (142 mg, 438 μmol) in CH_2Cl_2 (3.5 mL) was added trifluoroacetic acid (0.5 mL) at 0 °C. After stirring for 2 h at the same temperature, the starting **11** completely disappeared as judged from TLC study (R_f = 0.41, CH_2Cl_2/MeOH = 9/1), and the mixture was concentrated under reduced pressure to give crude 2-[2-(6-chlorohexyloxy)ethoxy]ethylammonium trifluoroacetate (**12**) as a colorless oil.

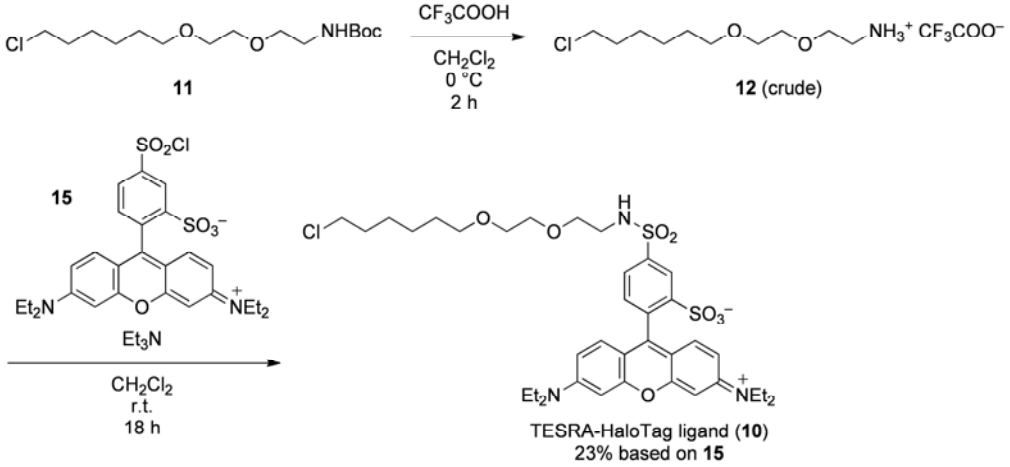
Under argon atmosphere, to a solution of succinimido 4-(azidomethyl)benzoate (**13**) (100 mg, 365 μmol) in CH_2Cl_2 (2 mL) were successively added triethylamine (153 μL, 1.09 mmol) and a solution of crude **12** prepared as above in CH_2Cl_2 (1 mL) at room temperature. After stirring for 18 h at the same temperature, to the mixture was added water (15 mL) and the product was extracted with CH_2Cl_2 (×3). The combined organic extracts were washed with water (×1) and dried over Na_2SO_4. After filtration, the filtrate was concentrated under reduced pressure and the residue was purified by flash column chromatography (silica-gel 10 g, *n*-hexane/EtOAc = 1/1) to give 4-(azidomethyl)-*N*-[2-{2-(6-chlorohexyloxy)ethoxy}ethyl]benzamide (**8**) (136 mg, 355 μmol, 97.3% based on **13**) as a yellow oil; R_f = 0.29 (*n*-hexane/EtOAc = 1/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.28–1.50 (m, 4H), 1.51–1.66 (m, 2H), 1.67–1.82 (m, 2H), 3.46 (t, 2H, *J* = 6.7 Hz), 3.52 (t, 2H, *J* = 6.7 Hz), 3.57–3.62 (m, 2H), 3.64–3.71 (m, 6H), 4.40 (s, 2H), 6.75 (br s, 1H), 7.36–7.42 (AA'BB', 2H), 7.79–7.85 (AA'BB', 2H); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 25.3, 26.6, 29.3, 32.4, 39.6, 45.0, 54.1, 69.6, 69.9, 70.1, 71.2, 127.5 (2C), 128.0 (2C), 134.4, 138.7, 166.8; IR (KBr, cm^{-1}) 557, 650, 733, 754, 853, 908, 1018, 1115, 1200, 1252, 1300, 1350, 1456, 1504, 1541, 1614, 1643, 2099, 2862, 2936, 3065, 3331; HRMS (EI) *m*/z 382.1775 (M, $C_{18}H_{27}{}^{35}ClN_4O_3$ requires 382.1772).

SS3-2. Synthesis of TESRA-PEO_3-azide (**9**).



Under argon atmosphere, to a solution of lissamine rhodamine B sulfonyl chloride (**15**) (600 mg, 1.04 mmol) in CH_2Cl_2 (30 mL) were successively added triethylamine (291 μL, 2.08 mmol) and 11-azido-3,6,9-trioxaundecan-1-amine (**14**) (≥90%) (275 μL, ≥1.25 mmol) at 0 °C. The mixture was allowed to warm to room temperature and stirred for 22 h at the same temperature. The mixture was concentrated under reduced pressure and the residual solid was placed on a funnel and then washed with EtOAc. The collected solid was purified by flash column chromatography (silica-gel 50 g, CH_2Cl_2/MeOH = 19/1) to give TESRA-PEO_3-azide (**9**) (421 mg, 554 μmol, 53.4%) as a purple solid; R_f = 0.61 (CH_2Cl_2/MeOH = 6/1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.20 (t, 12H, J = 7.1 Hz), 3.01 (br s, 2H), 3.32–3.68 (m, 22H), 6.86–7.07 (m, 6H), 7.46 (d, 1H, J = 7.7 Hz), 7.94 (d, 1H, J = 7.7 Hz), 8.06 (br s, 1H), 8.40 (s, 1H); IR (KBr, cm^{-1}) 613, 683, 1024, 1074, 1134, 1182, 1197, 1248, 1281, 1341, 1352, 1420, 1466, 1526, 1595, 1647; UV (MeOH) λ_{max} (logε) 560.5 nm (5.16); FL (MeOH) λ_{max} Em. 577 nm (Ex. 450 nm); HRMS (ESI$^+$) m/z 781.2649 ([M+Na]$^+$, $C_{35}H_{46}N_6NaO_9S_2^+$ requires 781.2660).

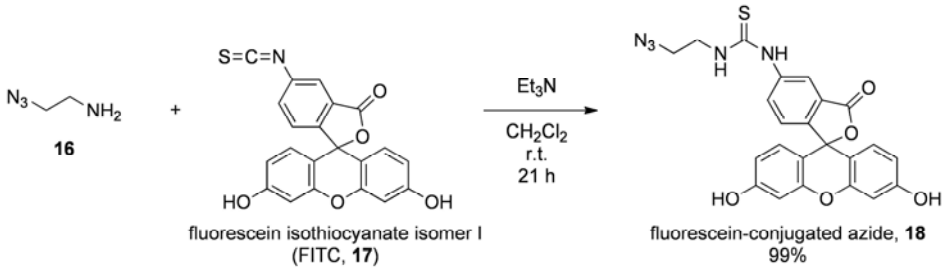
SS3-3. Synthesis of TESRA-HaloTag ligand (**10**).



Under argon atmosphere, to a solution of *tert*-butyl *N*-[2-{2-(6-chlorohexyloxy)ethoxy}ethyl]carbamate (**11**) (84.2 mg, 260 μmol) in CH_2Cl_2 (2.5 mL) was added trifluoroacetic acid (0.5 mL) at 0 °C. After stirring for 2 h at the same temperature, the starting **11** completely disappeared as judged from TLC study (R_f = 0.41, CH_2Cl_2/MeOH = 9/1), and the mixture was concentrated under reduced pressure to give crude 2-[2-(6-chlorohexyloxy)ethoxy]ethylammonium trifluoroacetate (**12**) as a colorless oil.

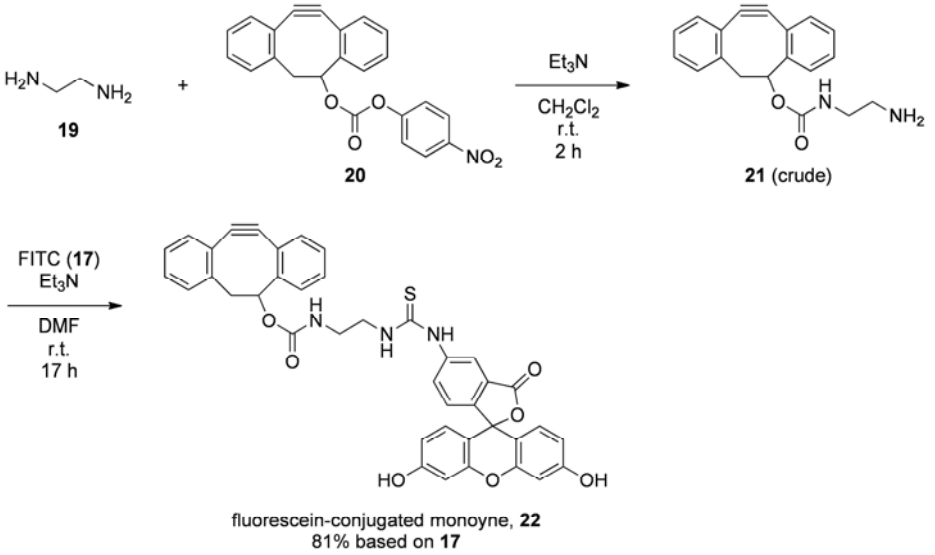
Under argon atmosphere, to a solution of lissamine rhodamine B sulfonyl chloride (**15**) (100 mg, 173 μmol) in CH_2Cl_2 (2 mL) were successively added triethylamine (72.4 μL, 519 μmol) and a solution of crude **12** as prepared above in CH_2Cl_2 (1.5 mL) at room temperature. After stirring for 18 h at the same temperature, the mixture was concentrated under reduced pressure and the residual solid was placed on a funnel and then washed with EtOAc. The collected solid was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2/MeOH = 15/1) to give TESRA-HaloTag ligand (**10**) (30.1 mg, 39.8 μmol, 22.8% based on **15**) as a purple solid; R_f = 0.54 (CH_2Cl_2/MeOH = 6/1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 1.20 (t, 12H, J = 7.0 Hz), 1.25–1.40 (m, 4H), 1.42–1.52 (m, 2H), 1.62–1.73 (m, 2H), 3.01 (br s, 2H), 3.42–3.51 (m, 8H), 3.55–3.70 (m, 10H), 6.90–7.10 (m, 6H), 7.45 (d, 1H, J = 7.7 Hz), 7.94 (d, 1H, J = 7.7 Hz), 8.06 (br s, 1H), 8.40 (s, 1H); IR (KBr, cm^{-1}) 579, 683, 1026, 1076, 1136, 1165, 1182, 1202, 1258, 1277, 1350, 1396, 1420, 1466, 1483, 1524, 1597, 1645; UV (MeOH) λ_{max} (logε) 561 nm (5.15); FL (MeOH) λ_{max} Em. 577 nm (Ex. 450 nm); HRMS (FAB$^+$/NBA) *m*/z 764.2834 (M+H, $C_{37}H_{51}{}^{35}ClN_3O_8S_2$ requires 764.2806).

SS3-4. Synthesis of fluorescein-conjugated azide **18**.



Under argon atmosphere, to a solution of 2-azidoethylamine (**16**) (~50%, ^{1}H NMR) (200 mg, ~1.16 mmol) in CH_2Cl_2 (5 mL) were successively added triethylamine (70.0 μL, 502 μmol) and fluorescein isothiocyanate isomer I (FITC, **17**) (≥90%, HPLC) (100 mg, ≥231 μmol) at room temperature. After stirring for 21 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 30 g, CH_2Cl_2/MeOH = 9/1) to give 1-(2-azidoethyl)-3-(5-fluoresceinyl)thiourea (**18**) (109 mg, 229 μmol, 99.2%) as an orange solid; TLC R_f = 0.61 (CH_2Cl_2/MeOH = 6/1); ^{1}H NMR (300 MHz, DMSO-d_6) δ 2.73 (t, 2H, J = 5.8 Hz), 3.56 (t, 2H, J = 5.8 Hz), 3.65–3.80 (br, 2H), 6.54 (dd, 2H, J = 2.2, 8.6 Hz), 6.61 (d, 2H, J = 8.6 Hz), 6.65 (d, 2H, J = 2.2 Hz), 7.18 (d, 1H, J = 8.4 Hz), 7,72 (dd, 1H, J = 1.7, 8.4 Hz), 8.21 (d, 1H, J = 1.7 Hz), 8.25–8.40 (br, 1H), 9.80–10.60 (br, 1H); IR (KBr, cm^{-1}) 459, 476, 577, 600, 667, 812, 851, 914, 1111, 1173, 1207, 1296, 1389, 1458, 1570, 2108, 2930; UV (MeOH) λ_{max} (logε) 456 nm (4.07), 482 nm (4.09); FL (MeOH) λ_{max} Em. 516.5 nm (Ex. 450 nm); HRMS (ESI$^+$) *m*/*z* 476.1024 ([M+H]$^+$, $C_{23}H_{18}N_5O_5S^+$ required 476.1023).

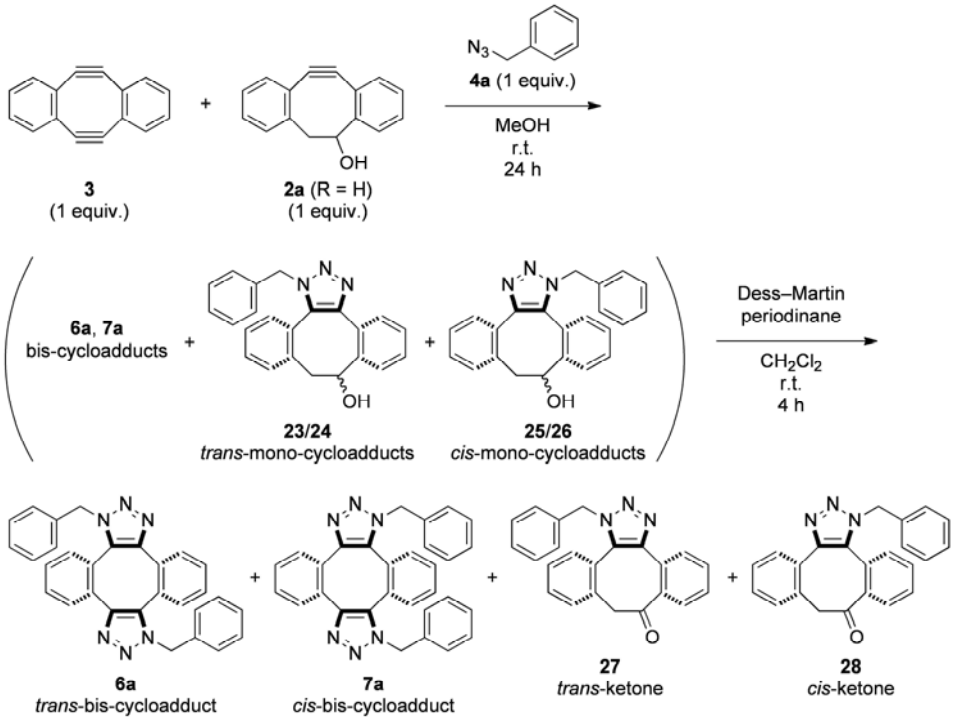
SS3-5. Synthesis of fluorescein-conjugated monoyne **22**.



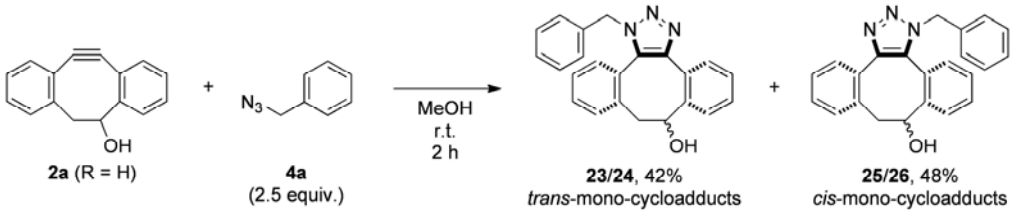
Under argon atmosphere, to a solution of ethylenediamine (**19**) (320 μL, 4.79 mmol) in CH_2Cl_2 (5 mL) were successively added triethylamine (330 μL, 2.37 mmol) and 11,12-didehydro-5,6-dihydrodibenzo[*a*,*e*]cycloocten-5-yl 4-nitrophenyl carbonate (**20**) (60.3 mg, 156 μmol) at room temperature. After stirring for 2 h at the same temperature, the starting **20** completely disappeared as judged from the TLC study. To this was added CH_2Cl_2 (70 mL) and the mixture was washed with saturated aqueous $NaHCO_3$ solution (20 mL ×7), dried over Na_2SO_4, filtered, and concentrated under reduced pressure to give crude 11,12-didehydro-5,6-dihydrodibenzo[*a*,*e*]cycloocten-5-yl *N*-(2-azidoethyl)carbamate (**21**) (51.2 mg) as a pale yellow oil, which was used in the next step without further purification.

Under argon atmosphere, to a solution of the crude **21** obtain as above in DMF (3 mL) were successively added triethylamine (29.0 μL, 208 μmol) and fluorescein isothiocyanate isomer I (FITC, **17**) (≥90%, HPLC) (45.0 mg, ≥104 μmol) at room temperature. After stirring for 17 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 8 g, CH_2Cl_2/MeOH = 12/1) to give 1-[2-{(11,12-didehydro-5,6-dihydrodibenzo[*a*,*e*]cycloocten-5-yl)oxycarbonylamino}ethyl]-3-(5-fluore sceinyl)thiourea (**22**) (59.0 mg, 84.8 μmol, 80.7% based on **17**) as a yellow solid; TLC R_f = 0.52 (CH_2Cl_2/MeOH = 6/1); ^{1}H NMR (300 MHz, CD_3OD) δ 2.73 (dd, 1H, *J* = 3.7, 14.9 Hz), 3.20 (dd, 1H, *J* = 2.4, 14.9 Hz), 3.70–3.80 (m, 2H), 5.28 (s, 1H), 6.41 (dd, 2H, *J* = 2.3, 9.2 Hz), 6.59 (dd, 2H, *J* = 2.3, 9.2 Hz), 6.70 (d, 1H, *J* = 2.3 Hz), 6.71 (d, 1H, *J* = 2.3 Hz), 6.97–7.56 (m, 10H), 7.83 (s, 1H) (two protons could not identified due to overlapping with the solvent peak); IR (KBr, cm^{-1}) 488, 546, 565, 578, 760, 851, 914, 993, 1022, 1076, 1113, 1179, 1207, 1258, 1317, 1449, 1506, 1595, 1701, 2938, 3063, 3287; UV (MeOH) λ_{max} (logε) 455 nm (3.95), 481 nm (3.95); FL (MeOH) λ_{max} Em. 515 nm (Ex. 450 nm); HRMS (ESI^+) *m*/*z* 696.1785 ($[M+H]^+$, $C_{40}H_{30}N_3O_7S^+$ required 696.1799).

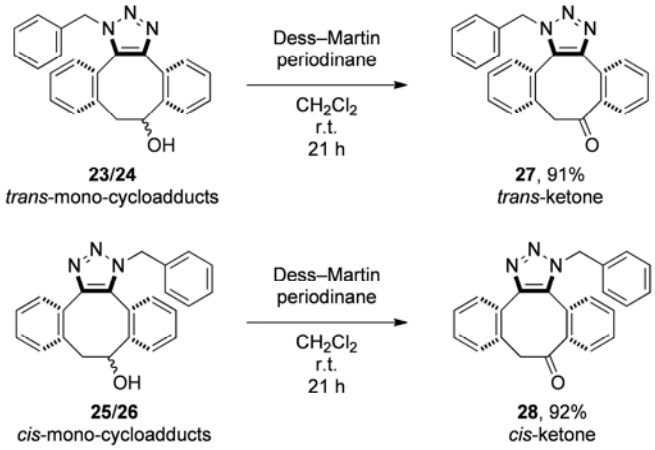
SS4. The competition between the SPDC reaction of diyne **3** and the single SPAAC reaction of monoyne **2a** (R = H) with benzyl azide (**4a**).



To a mixture of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) (20.0 mg, 100 μmol) and 11,12-didehydro-5,6-dihydrodibenzo[*a,e*]cycloocten-5-ol (**2a**, R = H) (22.0 mg, 100 μmol) dissolved in MeOH (22.5 mL) was added a solution of benzyl azide (**4a**) (13.3 mg, 100 μmol) in MeOH (2.5 mL) at room temperature. After stirring for 24 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was a mixture of two bis-cycloadducts **6a**/**7a** and four isomers of mono-cycloadducts **23**–**26**, which was difficult to separate chromatographically or determine the ratio of bis- and mono-cycloadducts by ^{1}H NMR. Thus the mixture of crude products was dissolved in CH_2Cl_2 (10 mL) and treated with Dess–Martin periodinane (37.2 mg, 87.7 μmol) at room temperature. After stirring for 4 h at the same temperature, the mixture was concentrated under reduced pressure. To remove the remaining oxidant, the residue was passed through a short silica-gel column (5 g, EtOAc only) to give a mixture of bis-cycloadducts **6a**/**7a** and ketones **27**/**28**. By comparing the integration values of protons on ^{1}H NMR spectrum, the molar ratio of bis-cycloadducts/mono-cycloadducts was determined to be approximately 1:1 indicating the similar reactivity of diyne **3** and monoyne **2a** (R = H) toward benzyl azide (**4a**).



For the purpose to prepare authentic samples of ketones **27** and **28**, to a solution of 11,12-didehydro-5,6-dihydrodibenzo[*a*,*e*]cycloocten-5-ol (**2a**, R = H) (108 mg, 490 μmol) in MeOH (11 mL) was added a solution of benzyl azide (**4a**) (163 mg, 1.22 mmol) in MeOH (4 mL) at room temperature. After stirring for 2 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by flash column chromatography (silica-gel 10 g, CH_2Cl_2/MeOH = 150/1 to 9/1) to give a mixture of *trans*-mono-cycloadducts **23**/**24** (R_f = 0.14 (CH_2Cl_2/MeOH = 49/1)) (72.8 mg, 206 μmol, 42.0% based on azide **4a**) and a mixture of *cis*-mono-cycloadducts **25**/**26** (R_f = 0.22 (CH_2Cl_2/MeOH = 49/1)) (83.0 mg, 235 μmol, 47.9% based on azide **4a**).

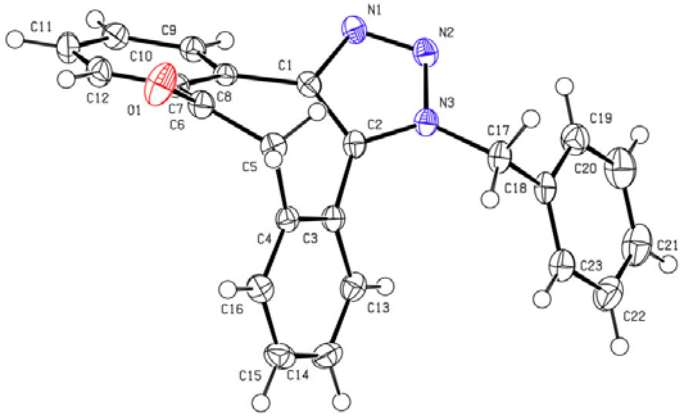


To a solution of the mixture of *trans*-mono-cycloadducts **23**/**24** (25.8 mg, 73.0 μmol) in CH_2Cl_2 (10 mL) was added Dess–Martin periodinane (37.2 mg, 87.7 μmol) at room temperature. After stirring for 21 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by successive flash column chromatography (silica-gel 10 g, EtOAc only, and then silica-gel 10 g, CH_2Cl_2 only) to give ketone **27** (23.3 mg, 66.3 μmol, 90.8%).

On the other hand, to a solution of the mixture of *cis*-mono-cycloadducts **25**/**26** (22.3 mg, 63.1 μmol) in CH_2Cl_2 (10 mL) was added Dess–Martin periodinane (32.1 mg, 75.7 μmol) at room temperature. After stirring for 21 h at the same temperature, the mixture was concentrated under reduced pressure. The residue was purified by successive flash column chromatography (silica-gel 10 g, EtOAc only, and then silica-gel 10 g, CH_2Cl_2 only) to give ketone **28** (20.5 mg, 58.3 μmol, 92.5%).

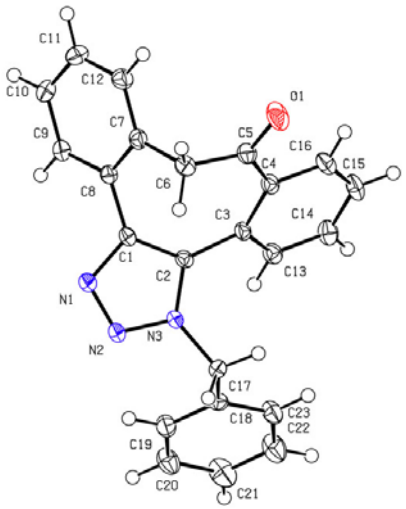
The geometries of **27** and **28** were confirmed by X-ray crystallographical analyses (CCDC 761157 (**27**) and CCDC 761156 (**28**)).

1-Benzyl-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazol-8(9*H*)-one (**27**)



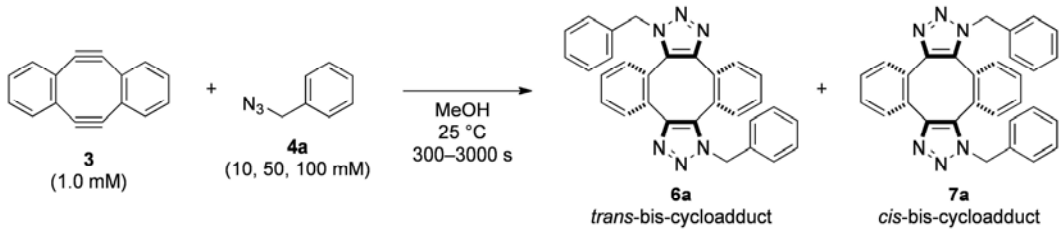
Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 220–222 °C; R_f = 0.55 (CH_2Cl_2/MeOH = 9/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.66 (d, 1H, *J* = 12.1 Hz), 3.76 (d, 1H, *J* = 12.1 Hz), 5.57 (d, 1H, *J* = 15.1 Hz), 5.69 (d, 1H, *J* = 15.1 Hz), 7.03–7.10 (m, 2H), 7.23–7.37 (m, 5H), 7.38–7.52 (m, 3H), 7.64 (ddd, 1H, *J* = 1.4, 8.0, 8.0 Hz), 8.01 (dd, 1H, *J* = 1.4, 8.0 Hz), 8.29 (dd, 1H, *J* = 1.4, 8.0 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 48.0, 52.5, 125.4, 127.2 (2C), 127.5, 128.2, 128.29, 128.34 (3C), 128.8, 129.8, 130.8, 130.9, 131.3, 133.0, 133.2, 133.8, 134.0, 135.0, 146.6, 195.4; IR (KBr, cm^{-1}) 542, 603, 704, 735, 764, 908, 1013, 1150, 1207, 1256, 1279, 1346, 1431, 1454, 1497, 1597, 1668, 3063.

1-Benzyl-1*H*-dibenzo[3,4:7,8]cycloocta[1,2-*d*][1,2,3]triazol-9(8*H*)-one (**28**)



Recrystallized from *n*-hexane/EtOAc to give colorless crystals; Mp 178–180 °C; R_f = 0.67 (CH_2Cl_2/MeOH = 9/1); ^{1}H NMR (300 MHz, $CDCl_3$) δ 3.60 (br, 1H), 3.81 (br, 1H), 5.67 (AB d, 2H), 7.01–7.09 (m, 2H), 7.18 (dd, 1H, *J* = 1.7, 7.7 Hz), 7.24–7.30 (m, 3H), 7.32–7.41 (m, 3H), 7.43–7.56 (m, 2H), 7.66–7.74 (m, 1H), 8.15 (dd, 1H, *J* = 1.7, 7.7 Hz); ^{13}C NMR (75.5 MHz, $CDCl_3$) δ 49.4, 53.1, 126.3, 127.3 (2C), 127.8, 128.5, 128.7, 128.9 (2C), 129.4, 129.5, 129.6, 130.5, 131.2, 131.4, 132.4, 133.2, 134.5, 134.8, 136.1, 145.8, 196.9; IR (KBr, cm^{-1}) 538, 604, 706, 730, 766, 908, 1028, 1157, 1211, 1250, 1281, 1352, 1429, 1454, 1497, 1597, 1672, 3063.

SS5. The kinetic study for the SPDC reaction of diyne **3** with benzyl azide (**4a**).



The rate measurement of the first cycloaddition in the SPDC reaction was performed by monitoring the absorbance of diyne **3** in the presence of an excess amount of benzyl azide (**4a**).

To 1.5 mL of 2.0 mM (final concentration at 1.0 mM) solution of *sym*-dibenzo-1,5-cyclooctadiene-3,7-diyne (**3**) in MeOH placed in a quartz cuvette (10 mm light path, temperature kept at 25 °C by isothermal water circulating jacket) was added 1.5 mL of MeOH solution of benzyl azide (**4a**) in three different concentrations (20, 100 or 200 mM; final concentration at 10, 50 and 100 mM, respectively). The consumption of diyne **3** was monitored by UV spectroscopy focusing at 351.5 nm, a wavelength that characteristic absorbance for the diyne ($\log\varepsilon = 3.22$) is observed[S10] but almost no significant absorption for azide **4a** and bis-cycloadducts **6a**/**7a** (Fig. SS1).

The monitoring was continued for 300–3000 s and the experiments were repeated in triplicate for each concentration of azide **4a**. The observed absorbance data at 351.5 nm were plotted versus time and fitted to a first order exponential decay curve (Fig. SS2).

The pseudo-first order rate constants were determined by least-squares fitting of the data to a single exponential equation using KaleidaGraph ver. 4.1.1. The pseudo-first order rate constants were plotted versus concentration of azide **4a** and fitted to a straight line by linear regression method using Microsoft Office Excel 2007 (Fig. SS3).

The slope of the straight line, $(6.29 \pm 0.05\ \text{SE}) \times 10^{-2}\ \text{M}^{-1}\ \text{s}^{-1}$, indicates the second order rate constant for the first cycloaddition in the SPDC reaction of diyne **3** and benzyl azide (**4a**), which is the rate-determining step of the reaction.

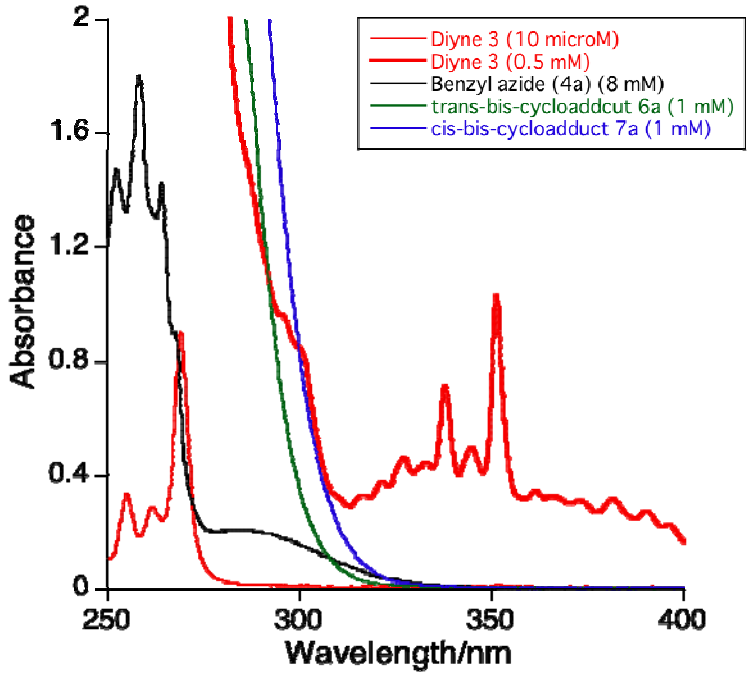
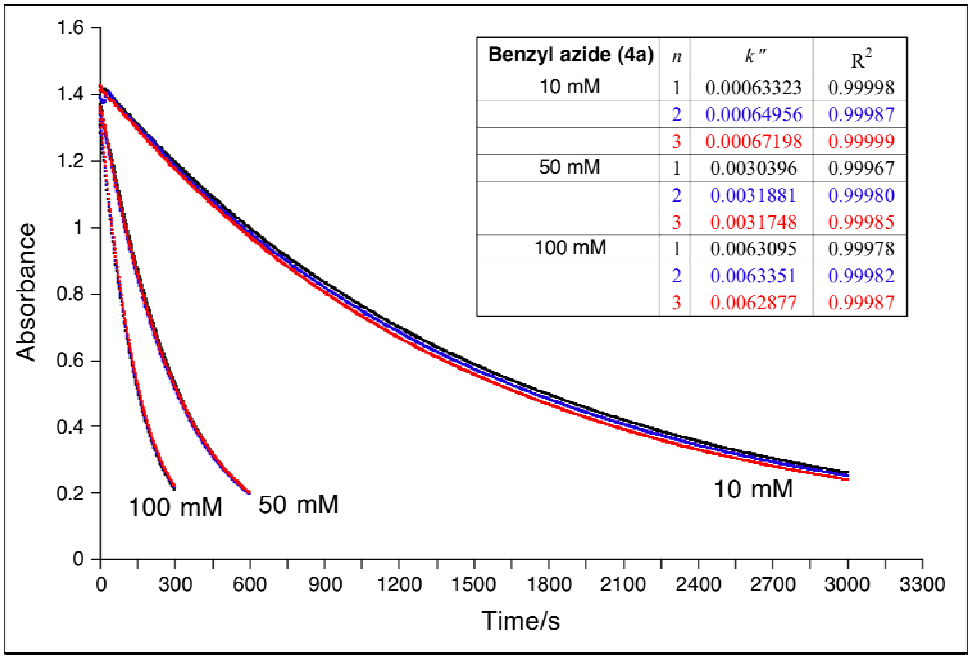


Fig. SS1 Absorption spectra of diyne **3** (10 μM and 0.5 mM), benzyl azide (**4a**, 8 mM) and bis-cycloadducts **6a**/**7a** (1 mM) in MeOH.



Benzyl azide (4a)	*n*	*k* ″	R^2
10 mM	1	0.00063323	0.99998
	2	0.00064956	0.99987
	3	0.00067198	0.99999
50 mM	1	0.0030396	0.99967
	2	0.0031881	0.99980
	3	0.0031748	0.99985
100 mM	1	0.0063095	0.99978
	2	0.0063351	0.99982
	3	0.0062877	0.99987

Fig. SS2 Time-dependent absorbance of diyne **3** (initial concentration of 1.0 mM) at 351.5 nm of wavelength monitored during the reaction with benzyl azide (**4a**) in three different concentrations (10, 50 or 100 mM).

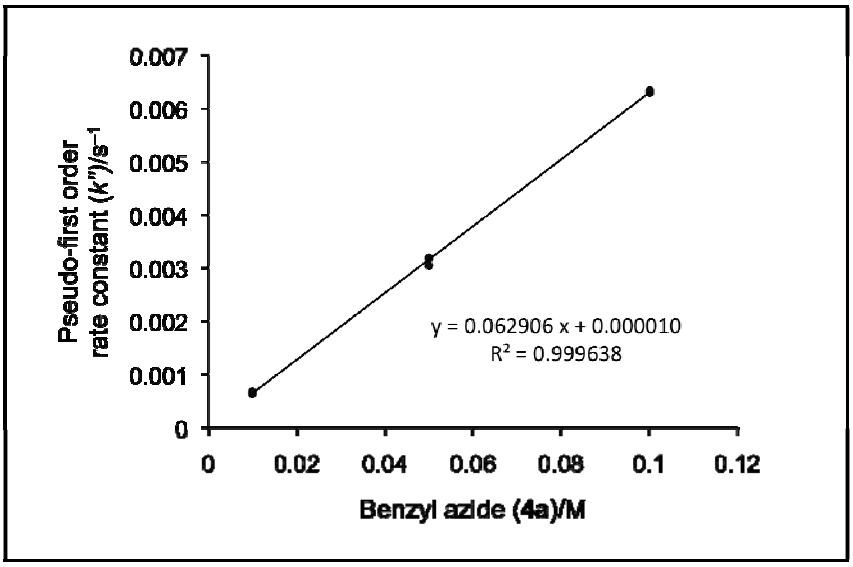
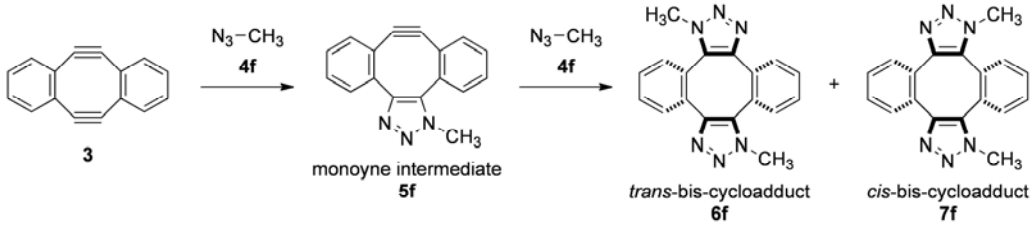


Fig. SS3 A plot of pseudo-first order rate constants (*k*") versus the concentration of benzyl azide (**4a**).

SC. Computations.

The results shown in Fig. 2 were obtained from a density functional theory (DFT) calculation using the GAMESS suite of program codes.[S11] Fig. 2 shows the relevant stationary points and transition states on the potential energy surfaces (PES) for each cycloaddition of the SPDC reaction. Geometry optimizations were carried out using the B3LYP functional with the 6-31G(d) basis set. All energies include zero-point energy corrections at the level used for geometry optimization. Transition structures were confirmed to be true transition states on PES by achieving vibrational frequency analyses and intrinsic reaction coordinate approaches.

Table SC1 Distortion,[a] interaction[b] and activation energies[c] (in kcal mol^{-1}) for each cycloaddition in the SPDC reaction of diyne **3** with methyl azide (**4f**) at the B3LYP/6-31G(d) level without zero point energy.[S12–S19]



Reactants	**3** + **4f**	**5f** + **4f**	**5f** + **4f**
Product	monoyne, **5f**	*trans*-adduct, **6f**	*cis*-adduct, **7f**
Distorted reactants[d]	+3.1 +15.7	+1.9 +11.5	+2.0 +11.9
Distortion energy[a]	+18.7	+13.5	+14.0
Interaction energy[b]	–8.7	–7.4	–7.1
Activation energy[c]	+10.0	+6.1	+6.9

[a]The energy required to distort the geometries of reactants to those of the transition state.

[b]The interaction energy between the distorted fragments at the transition states.

[c]The activation energy shown here is smaller than that indicated in Fig. 2 because it dose not include the zero-point energy correlations, and the total energy of the reactant is obtained by sum of each reactant.

[d]The energy differences of each fragments between the optimized and the transition geometries.

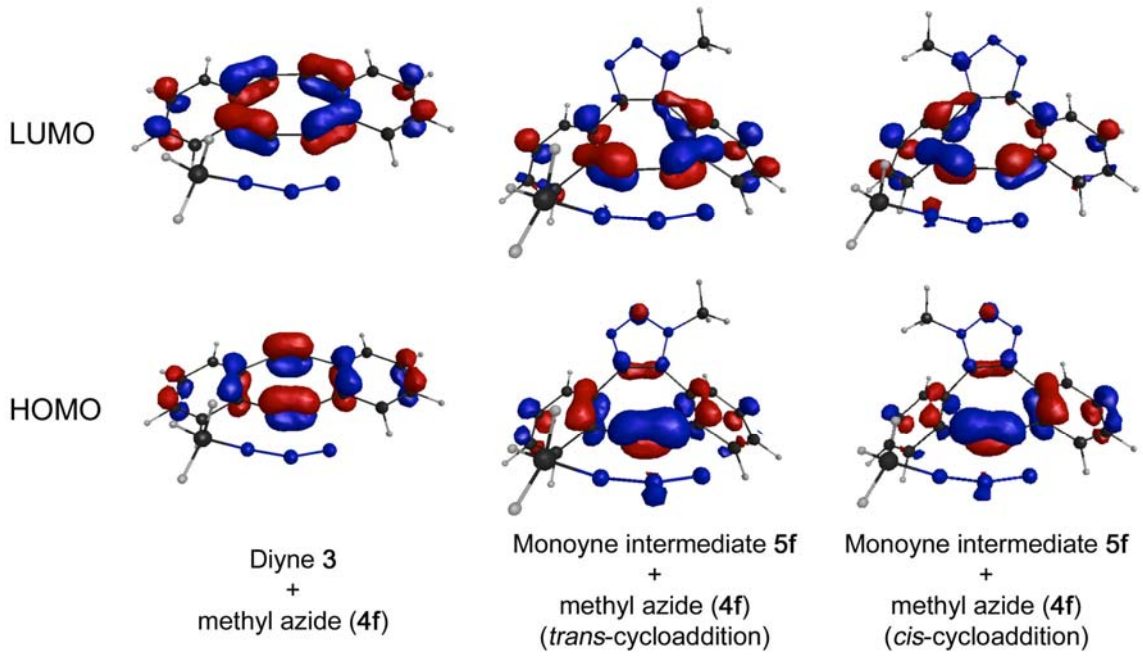


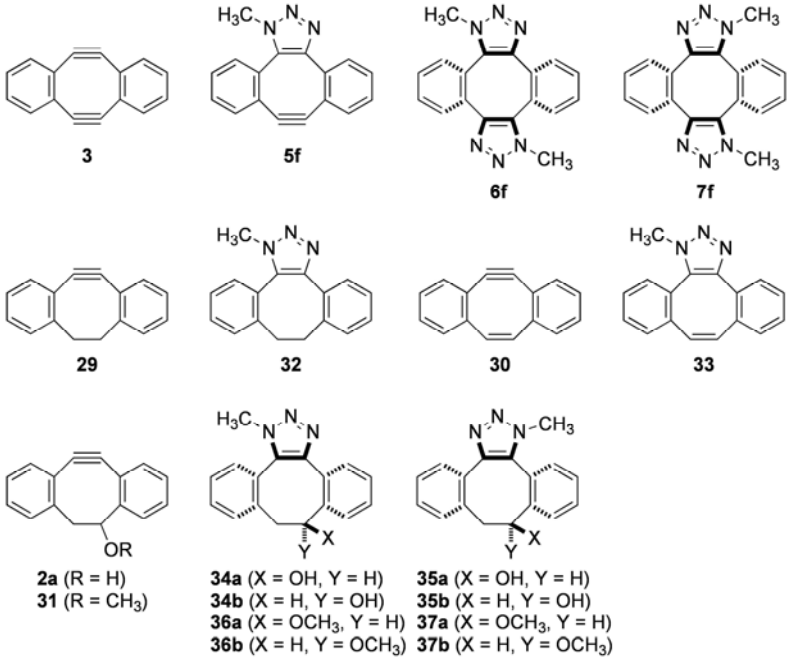
Fig. SC1 Kohn–Sham orbital amplitude plots for the HOMO (bottom) and LUMO (top) of transition structures for the first (left side) and the second (middle and right side) cycloadditions in the SPDC reaction of diyne **3** with methyl azide (**4f**).[S20] The orbital coefficients of the monoyne intermediate **5f** are localized at the alkyne carbons also confirming the faster reaction of the second cycloadditions. On the other hand, the orbital coefficients of diyne **3** are delocalized over the whole structure, supporting the slower reaction.

Table SC2 Relative energies of the transition state and the product for cycloadditions of diyne **3**, monoyne intermediate **5f** and some related monoynes, **2a** (R = H) and **29**–**31**, with methyl azide (**4f**), using B3LYP/6-31G(d) DFT method. All energies include zero-point energy corrections.

Reactants	Product	Relative energies (kcal mol^{-1}) Transition State	Product
3 + **4f**	monoyne, **5f**	+12.4[a]	–62.3[a]
5f + **4f**	*trans*-adduct, **6f**	+8.8[a]	–78.7[a]
5f + **4f**	*cis*-adduct, **7f**	+9.5[a]	–76.6[a]
29 + **4f**	**32**	+12.2 (+12.3)[b]	–66.4
30 + **4f**	**33**	+11.8	–74.8
2a (R =H) + **4f**	**34a**	+12.4	–69.4
2a (R =H) + **4f**	**34b**	+12.4	–65.7
2a (R =H) + **4f**	**35a**	+11.9	–67.1
2a (R =H) + **4f**	**35b**	+14.0	–65.9
31 + **4f**	**36a**	+12.1	–69.5
31 + **4f**	**36b**	+12.4	–65.4
31 + **4f**	**37a**	+12.1	–67.3
31 + **4f**	**37b**	+13.4	–65.5

[a]The results shown in Fig. 2.

[b]The value reported in ref. 17.



SB. Biological experiments.

SB1. DNA construction.

To construct HaloTag-GST fusion protein, we amplified HaloTag 7 open reading frame from pFC14A (Promega Corporation, Madison, WI) with KOD plus DNA polymerase (TOYOBO, Osaka, Japan). The PCR primers for HaloTag 7 open reading frame were as follow: 5'-GGAATTCGGATCCGAAATCGGTACTG-3' (Forward) and 5'-CCGCTCGAGCTATTAACCGGAAATCTCCAGAG-3' (Reverse). The amplified fragment was digested with EcoRI and XhoI, and then subcloned into EcoRI/XhoI site of pGEX6P-1 (GE Healthcare UK Ltd., Buckinghamshire, England), pGEX6P-1-HaloTag. The PCR product was sequenced by using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

SB2. Production of recombinant HaloTag-GST protein in *E. coli*.

Escherichia coli strain Rosetta (DE3) pLysS cells (Novagen, Merck Chemicals Ltd., Nottingham, England) were transformed with the pGEX6P-1-HaloTag vector, and cultured in LB media containing 100 mg L^{-1} ampicillin (Nacalai Tesque, Kyoto, Japan) and 50 mg L^{-1} chloramphenicol (Nacalai Tesque). Expression was induced by the addition of isopropyl β-D-thiogalactopyranoside (final concentration at 2 mM) (Nacalai Tesque), when the culture had reached an OD_{600} of approximately 0.8. After induction for 16 h at 22 °C, the cells were collected by centrifugation at 6000 *g* for 5 min, and suspended in cell lysis buffer containing 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, and 100 mM phenylmethylsulfonyl fluoride (Nacalai Tesque), and then frozen in liquid N_2. After thawing, Triton X-100 (final concentration at 0.1%) was added to the cell lysate, which were then incubated at 4 °C for 10 min. After gentle sonication of the lysate, $MgCl_2$ (final concentration at 1 mM) and DNase I (final concentration of approximately 10 μg mL^{-1}) were added to the cell lysate, and incubation was continued at 4 °C for 20 min. Cell debris and larger particles were removed by centrifugation at 9000 *g* for 30 min, and the supernatant was then filtered through a 0.45-μm filter. The supernatant of the cell lysate containing the HaloTag-GST protein was applied onto a GSH-Sepharose 4B resin (GE Healthcare Bio-Science AB, Uppsala, Sweden), which had been pre-equilibrated with 50 mM Tris-HCl (pH 7.0) buffer containing 150 mM NaCl. After excessive washing of the resin with the equilibrating buffer, the bound HaloTag-GST protein was subjected to the modification with diyne **3**.

To analyze the concentration of the HaloTag-GST protein, we performed SDS-PAGE. The protein sample was diluted 1:1 with 2× SDS sample loading buffer (0.12 M Tris-HCl, pH 6.8, containing 3.4% SDS, 10% glycerol and 20 mM DTT), heated at 95 °C for 5 min, and then loaded onto the gels. The proteins were stained with Coomassie brilliant blue (CBB) rapid stain kit (Nacalai Tesque). The concentration of the recombinant proteins was determined by using CBB solution for protein assays with bovine serum albumin (Fraction V; Nacalai Tesque) as the standard.

SB3. Chemical modification of the HaloTag protein by the SPDC reaction.

The HaloTag-GST (total 1.8 nmol in 1 mL of reaction mixture), bound on the GSH-Sepharose resin (bed volume; 10 μL), was incubated with or without 100 μM of azido-HaloTag ligand **8** in PBS containing 0.3% H_2O_2 overnight at 4 °C. The azide-incorporated HaloTag-GST protein bound on the resin (azido-HaloTag-GST-resin) was extensively washed with PBS, and then mixed with 200 μM of TESRA-PEO_3-azide (**9**) in PBS containing 10%DMSO. Diyne **3** was added into the mixture to final 200 μM, and the tube was incubated at room temperature for 15 min. The azido-HaloTag-GST-resin was extensively washed with PBS10%DMSO for the following SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analyses.

In the sequential SPDC modification, the azido-HaloTag-GST-resin was reacted with 200 μM of diyne **3** in PBS10%DMSO at room temperature for 15 min. After quick washes with PBS10%DMSO, the resin was reacted with 200 μM of TESRA-PEO_3-azide (**9**) in PBS10%DMSO at room temperature for 10 min. The resin was extensively washed with PBS10%DMSO. For SDS-PAGE, the labeled HaloTag-GST was eluted by incubation for 5 min at 95 °C with 1× SDS sample loading buffer.

For MALDI-TOF-MS analysis, the labeled HaloTag was excised from GST tag by the addition of PreScission protease (GE Healthcare Bio-Science AB) in 50 mM Tris-HCl, containing 150 mM NaCl, 1 mM DTT and 1 mM EDTA to the Sepharose resin, and incubation at 4 °C overnight. The recombinant protein was then eluted with 2 bed volumes of 50 mM Tris-HCl (pH 7.0) containing 150 mM NaCl. Then, the eluate was subjected to MALDI-TOF-MS analysis.

In the SPDC modification of soluble azido-HaloTag protein that was free from the resin, the HaloTag protein was excised from the HaloTag-GST-resin by PreScission protease, and dialyzed against tris-buffered saline. The soluble HaloTag protein (60 μM) was incubated with **8** (200 μM) and 0.3% H_2O_2 overnight at 4 °C. Unreacted **8** was removed by dialysis against PBS. The soluble azido-HaloTag protein (20 μM) was incubated with **3** (40 μM) at room temperature for the indicated times (0, 5, 10, 20, 40, 80 min) in PBS10%DMSO. After the incubation, **9** was immediately added to the reaction mixture at final 200 μM, and then incubated at room temperature for 20 min. The labeled proteins were incubated for 20 min at 70 °C with 1× SDS sample loading buffer.

SB4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE analysis was carried out under reducing conditions using a 10% separation gel, as described by Laemmli.[S21] The gels were directly visualized by laser-scanning in a fluorescence imaging analyzer Typhoon 8600 (GE Healthcare). The gels were also stained with CBB rapid stain kit (Nacalai Tesque).

SB5. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS).

MALDI-TOF MS was measured on an ultraflex P TOF/TOF mass spectrometer (Bruker Daltonics Inc., Billerica, MA). The accelerating voltage in the ion source was set to 25 kV. Data were acquired in the positive linear mode of operation. Time-to-mass conversion was achieved by external calibration using standards of trypsinogen (*m/z* 23982) and protein A (*m/z* 44613, 22307). The matrices for

proteins were sinapic acid (SA, Mw = 224; Bruker Daltonics Inc.). Saturated SA matrix solutions were prepared in a 30% (v/v) solution of acetonitrile in water containing 0.1% trifluoroacetic acid. The matrix (4 μL) was mixed with a solution (1 μL) of the labeled HaloTag proteins (0.7 μg μL^{-1}), and 1 μL of the mixture was applied on a steel sample plate (MTP 384 target plate polished steel; Bruker Daltonics Inc.). The mixture was allowed to air dry before being introduced into the mass spectrometer.

SB6. Cell culture.

HEK293 cells were routinely maintained in a 5% CO_2, water-saturated atmosphere, and grown in low-glucose Dulbecco's Modified Eagle Medium (DMEM; Nacalai Tesque) supplemented with 10% Fetal Bovine Serum (FBS; JRH Biosciences, Inc., Lenexa, KS), 100 units mL^{-1} of penicillin (Nacalai Tesque), and 100 μg mL^{-1} of streptomycin (Nacalai Tesque).

Phase contrast images for the cytotoxicity assay were collected on an inverted microscope (IX70; Olympus Corporation, Japan) equipped with 20× LCPlanFI NA 0.40 Ph1 objective lens (Olympus Corporation) and DP11-N charge coupled device (CCD) camera (Olympus Optical Co., Ltd., Japan). Images were imported into Photoshop (Ver. CS2; Adobe) for cropping and linear contrast adjustment.

SB7. Fluorescence labeling of glycoconjugates in living cells.

Fifty microliter of Ac_4ManNAz (Sigma-Aldrich; 1 mM in ethanol) was added onto 1.2 cm-diameter coverslips (Matsunami Glass Ind., Ltd, Osaka, Japan) in a 24-well cell culture plate (Corning Incorporated, Corning, NY), and then evaporated in the clean bench at room temperature. HEK293 cells were cultured on the coverslips with 500 μL of DMEM10%FBS containing antibiotics for 2 days, which therefore contained 100 μM of Ac_4ManNAz. The azidosugar-incorporated cells were washed with pre-warmed DMEM10%FBS once, and incubated with 2 to 100 μM of diyne **3** for the indicated time (3 to 60 min) at 37 °C. After a wash with pre-warmed DMEM10%FBS, the cells were incubated with 0 to 40 μM of TESRA-PEO_3-azide (**9**), Alexa Fluor 488 azide (Molecular probes, Invitrogen Corporation), or fluorescein-conjugated azide **18** for 10 or 20 min at 37 °C. The azidosugar-incorporated cells were also incubated with 40 μM of fluorescein-conjugated monoyne **22** for 10 or 20 min at 37 °C. After a wash with pre-warmed DMEM10%FBS, the cells were incubated with TO-PRO-3 (Molecular probes, Invitrogen Corporation) to stain nuclei. The stained cells, incubated with phenol red-free low-glucose DMEM (GIBCO, Invitrogen Corporation) containing 5% FBS and 50 mM HEPES (pH 7.4), were observed on a laser-scanning confocal microscopy (FV1000-BX61; Olympus Corporation) with 60× LUMPlanFI NA 0.90 objective lens (water immersion) using imaging software (FLUOVIEW Ver. 1.6a; Olympus Corporation). To visualize cellular cytoskeleton, the labeled cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) in PBS for 10 min at room temperature. Residual free aldehyde groups were blocked by incubation with 10 mg/mL of glycine (Nacalai Tesque) in PBS for 20 min at room temperature. After an incubation with 1% bovine serum albumin (Fraction V; Nacalai Tesque) in PBS, which was pre-heated at 55 °C for 30 min, for 20 min at room temperature, the fixed cells were incubated with Alexa Fluor 488-phalloidin

(Molecular probes, Invitrogen Corporation) and TO-PRO-3 for 60 min at room temperature. After a wash with PBS followed by a wash with ultra pure water, the coverslips were mounted on a slide glass (Matsunami Glass Ind., Ltd.) with SlowFade Gold antifade reagent (Invitrogen Corporation). Fluorescence images were collected on a laser-scanning confocal microscopy (FV1000-BX61; Olympus Corporation) with 40× UPlanApo NA 0.85 and 100× UPlanApo NA 1.35 objective lenses using imaging software (FLUOVIEW Ver. 1.6a; Olympus Corporation). Images were imported into Photoshop (Ver. CS2; Adobe) for cropping and linear contrast adjustment. Fluorescence intensity was measured by the ImageJ 1.34s software (NIH, USA). Data were summarized as the mean ± SEM.

SB8. Supporting Figures.

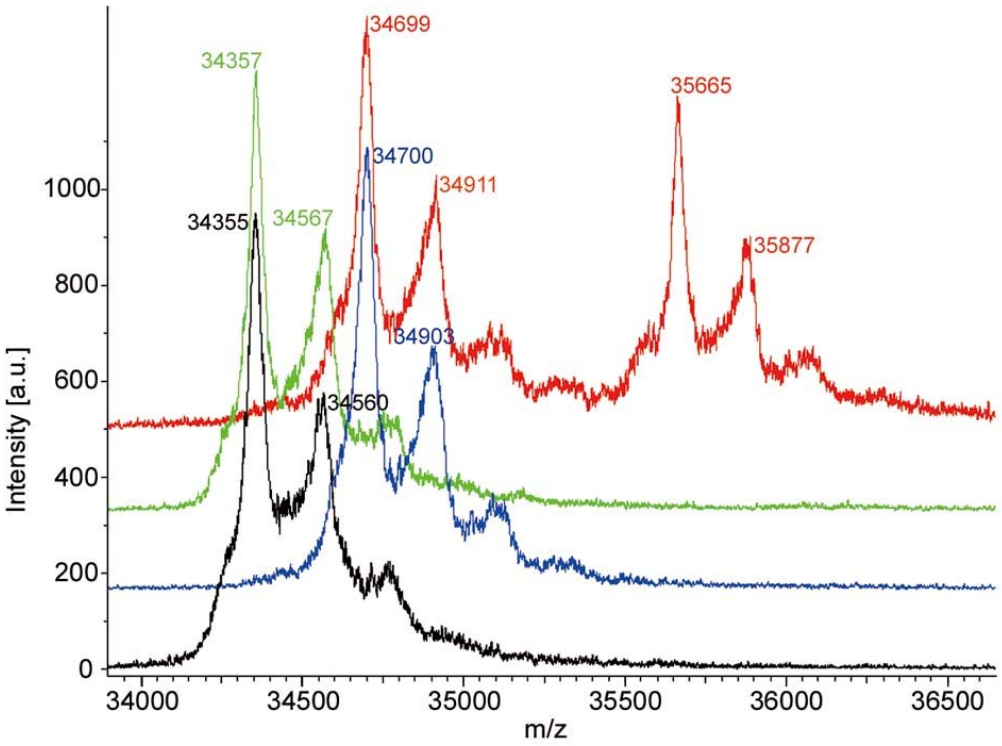


Fig. SB1 Mass spectra of the HaloTag proteins sequentially modified with azido-HaloTag ligand **8**, diyne **3** and TESRA-PEO_3-azide (**9**). The each modified HaloTag-GST, in which a cleavage site recognized by PreScission protease was located between the tags, was digested with the protease overnight at 4 °C to elute the modified HaloTag from the Sepharose resin. The eluted proteins were analyzed by MALDI-TOF-MS. Mass spectrum of the HaloTag is shown as a black line; the HaloTag reacted with **3** and **9**, green line; the HaloTag reacted with **8**, blue line; the HaloTag reacted with **8**, **3** and **9**, red line. The mass of unlabeled HaloTag observed at *m/z* 34355 as a major peak matched with its calculated value of 34336 (black line). The accompanied minor peak at *m/z* 34560 presumably arose from a complex of the HaloTag and sinapic acid (Mw = 224) used as a matrix assisting the ionization (black line). This matrix-adducted peak was observed in every case. After incorporation of azido-HaloTag ligand **8** (Mw = 383) into the HaloTag, the major peak shifted to a mass of 34700, which corresponds to the azido-HaloTag (Calcd. Mw = 34684) (blue line). Mass analysis after the SPDC reaction of the azido-HaloTag with diyne **3** (Mw = 200) and TESRA-PEO_3-azide (**9**) (Mw = 759) showed two major peaks at *m/z* 34699 and 35665, which corresponds to the unreacted azido-HaloTag and SPDC product (Calcd. Mw = 35643), respectively (red line). Thus, increases of mass at each step were in good agreement with the calculated mass values of the modified HaloTag proteins. In addition, the observed mass spectrum of the HaloTag incubated with diyne **3** and TESRA-PEO_3-azide (**9**) without azido-HaloTag ligand **8** (green line) was almost the same with that of the unlabeled HaloTag (black line), indicating that neither diyne **3** nor TESRA-PEO_3-azide (**9**) reacted with amino acid residues of the HaloTag protein.

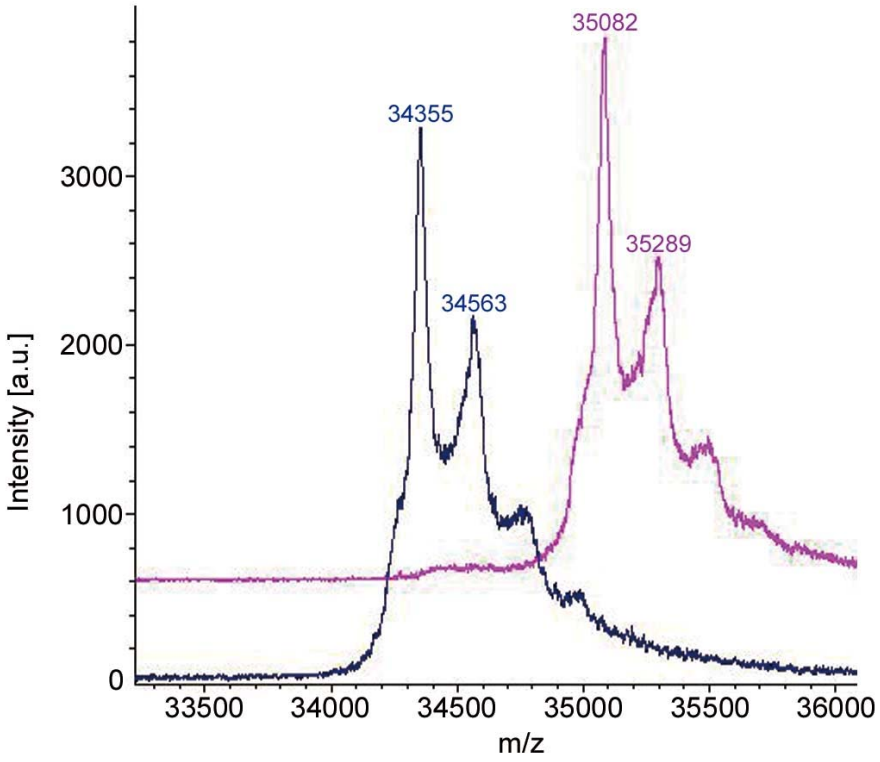


Fig. SB2 Mass spectra of the HaloTag protein modified with TESRA-HaloTag ligand (**10**). The modified HaloTag-GST was digested with the protease overnight at 4 °C to elute the modified HaloTag from the Sepharose resin. The eluted protein was analyzed by MALDI-TOF-MS. Mass spectrum of the HaloTag is shown as a mazarine line; the HaloTag reacted with **10**, magenta line. The mass of unlabeled HaloTag observed at *m/z* 34355 as a major peak matched with its calculated value of 34336 (mazarine line). The accompanied minor peak at *m/z* 34563 presumably arose from a complex of the HaloTag and sinapic acid (Mw = 224) used as a matrix assisting the ionization (mazarine line). After labeling the HaloTag with TESRA-HaloTag ligand (**10**, Mw = 764), the major peak shifted to a mass of 35082, which corresponds to the TESRA-HaloTag (Calcd. Mw = 35065) (magenta line). The increase of mass was in good agreement with the calculated mass value of the modified HaloTag protein. By the addition of TESRA-HaloTag ligand (**10**), most of the peak of the starting HaloTag disappeared, indicating that the labeling mostly proceeded.

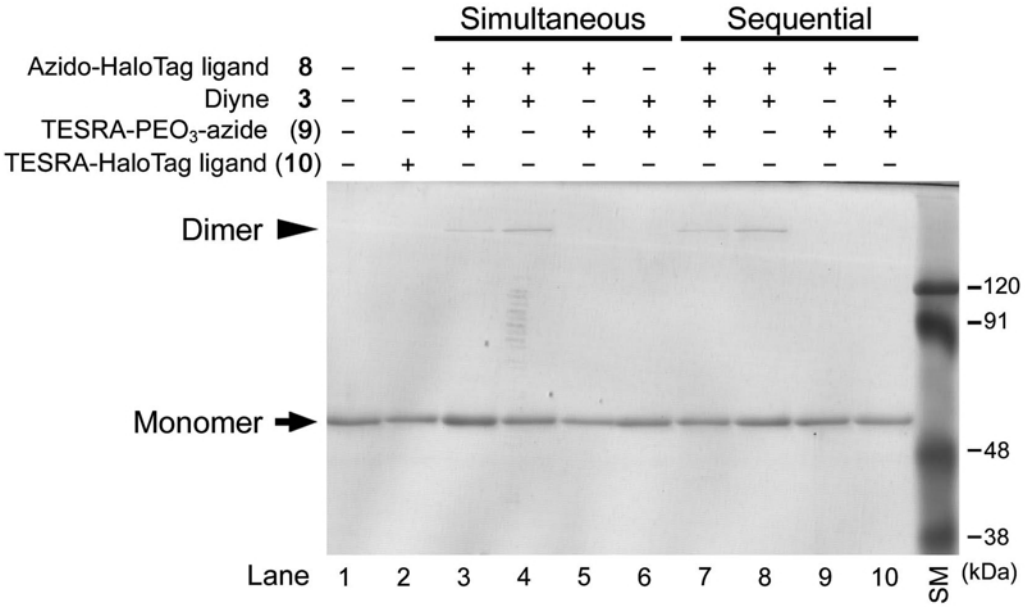


Fig. SB3 Diyne **3**-mediated dimerized product of the azido-HaloTag-GST protein bound on the resin is substantially undetectable. The entire image of the gel stained with Coomassie brilliant blue from the high- to low-molecular weight region shown in Fig. 3B. Arrow indicates the size of the HaloTag-GST protein monomer, and arrowhead indicates that corresponding to the dimer.

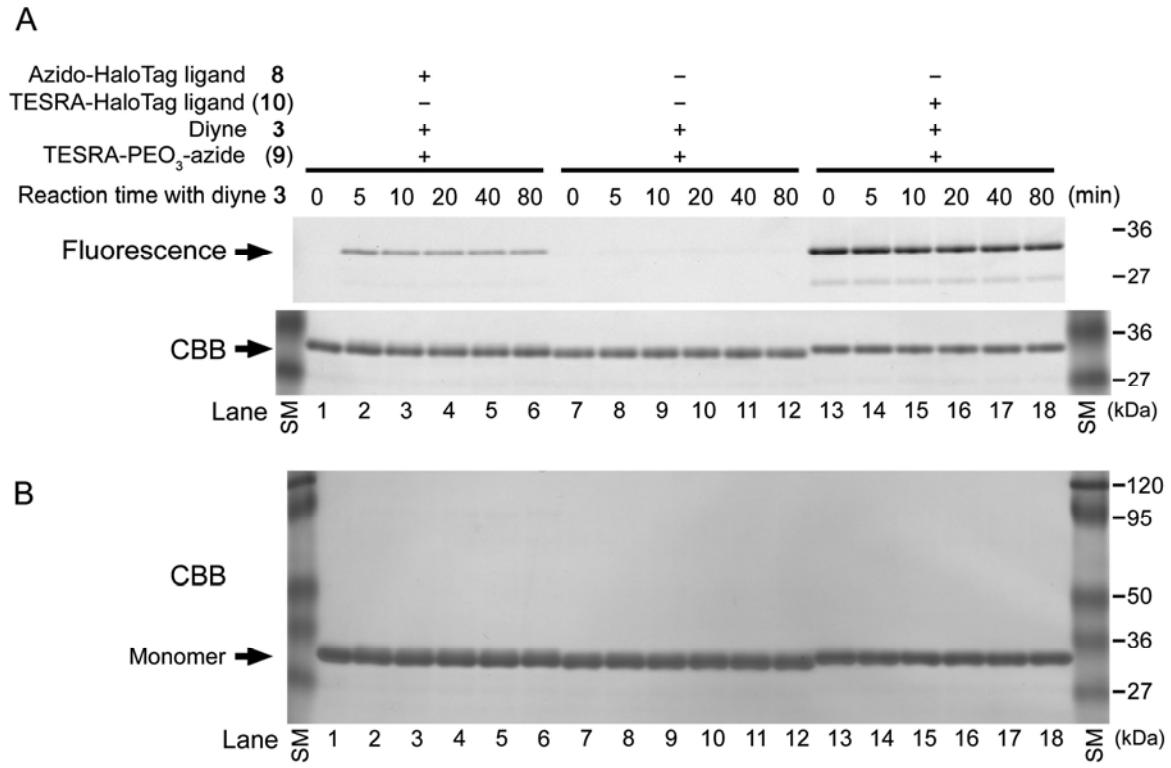


Fig. SB4 The SPDC modification of soluble azido-HaloTag protein that is free from the resin. (A) Purified HaloTag protein (60 μM) was treated with buffer (–), azido-HaloTag ligand **8** (200 μM) or TESRA-HaloTag ligand (**10**) (200 μM) overnight at 4 °C. The treated HaloTag proteins (20 μM) were incubated with diyne **3** (40 μM) for the indicated time at r.t., then TESRA-PEO_3-azide (**9**) was added into the reaction mixture at final 200 μM, and incubated for 20 min at r.t.. One hundred nanograms of the proteins were subjected to SDS-PAGE, and the gel was scanned with a fluorescence image analyzer (Typhoon 8600) and then stained with Coomassie brilliant blue (CBB). SM indicates size marker. (B) Five hundred nanograms of the proteins described above were subjected to SDS-PAGE, and the gel was stained with CBB. The entire image of the gel from the high- to low-molecular weight region is shown. The homo-dimer of the azido-protein was hard to detect.

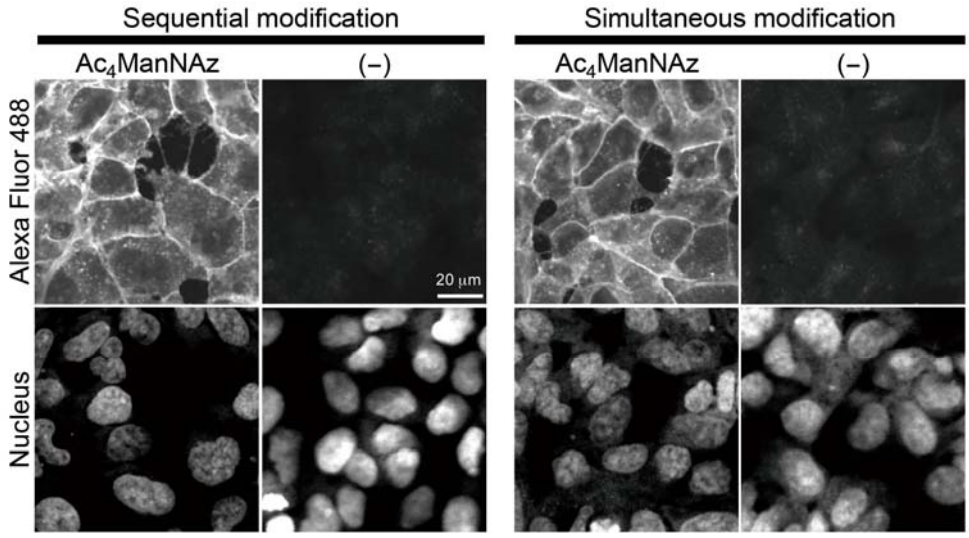


Fig. SB5 Cell-surface labeling with diyne **3** and Alexa Fluor 488 azide. HEK293 cells were incubated in the absence (–) or presence of 100 μM of Ac_4ManNAz for 2 days. The cells were incubated with 40 μM of diyne **3** for 10 min at 37 °C, and then with 40 μM of Alexa Fluor 488 azide for 10 min at 37 °C (Sequential modification). The cells were also incubated with 40 μM of diyne **3** and 40 μM of Alexa Fluor 488 azide for 20 min at 37 °C (Simultaneous modification). The labeled cells were stained with TO-PRO-3 to visualize nuclei.

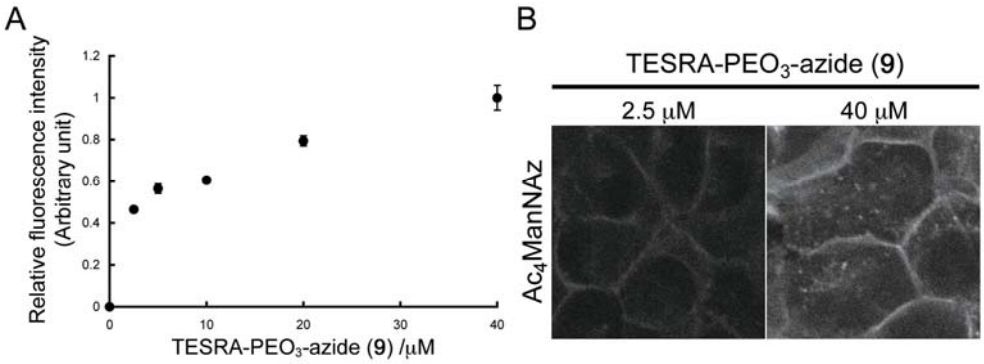


Fig. SB6 Dose-dependent increase of fluorescence intensity in the SPDC modification of azidosugar-incorporated glycoconjugates with TESRA-PEO_3-azide (**9**). (A) The azidosugar-incorporated HEK293 cells were incubated with 40 μM of diyne **3** for 10 min at 37 °C, and then with 0 to 40 μM of TESRA-PEO_3-azide (**9**) for 10 min at 37 °C. Relative fluorescence intensity was determined by densitometric analysis of the fluorescence images. (B) Fluorescence images of TESRA in the azidosugar-incorporated cells labeled with 40 μM of diyne **3** for 10 min at 37 °C, followed by with 2.5 μM or 40 μM of **9** for 10 min at 37 °C. Reliable fluorescence labeling was achieved at 2.5 μM of **9**; however, optimal results were obtained at concentrations ranging from 20 to 40 μM of **9**.

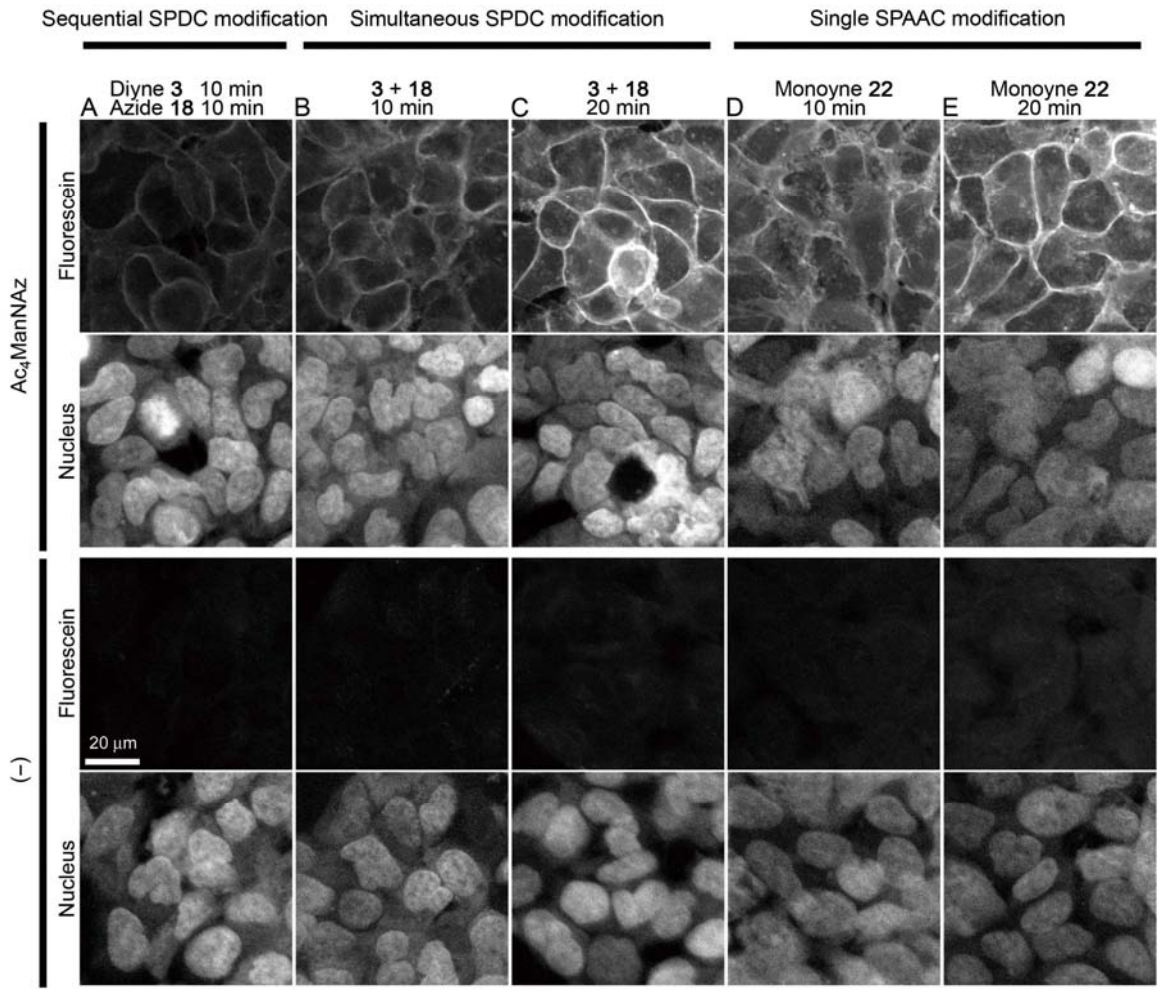


Fig. SB7 Comparison between the SPDC modification and the single SPAAC modification in the fluorescence labeling of azidosugar-incorporated glycoconjugates on the cell surface. HEK293 cells were incubated for 2 days in the absence (–) or presence of 100 μM of Ac_4ManNAz. (A) The azidosugar-incorporated cells were incubated with 40 μM of diyne **3** for 10 min at 37 °C, and then with 40 μM of fluorescein-conjugated azide **18** for 10 min at 37 °C. (B and C) The cells were incubated with 40 μM of **3** and 40 μM of **18** for 10 min (B) or 20 min (C). (D and E) The cells were incubated with 40 μM of fluorescein-conjugated monoyne **22** for 10 min (D) or 20 min (E). The labeled cells were fixed, and then stained with TO-PRO-3 to visualize nuclei.

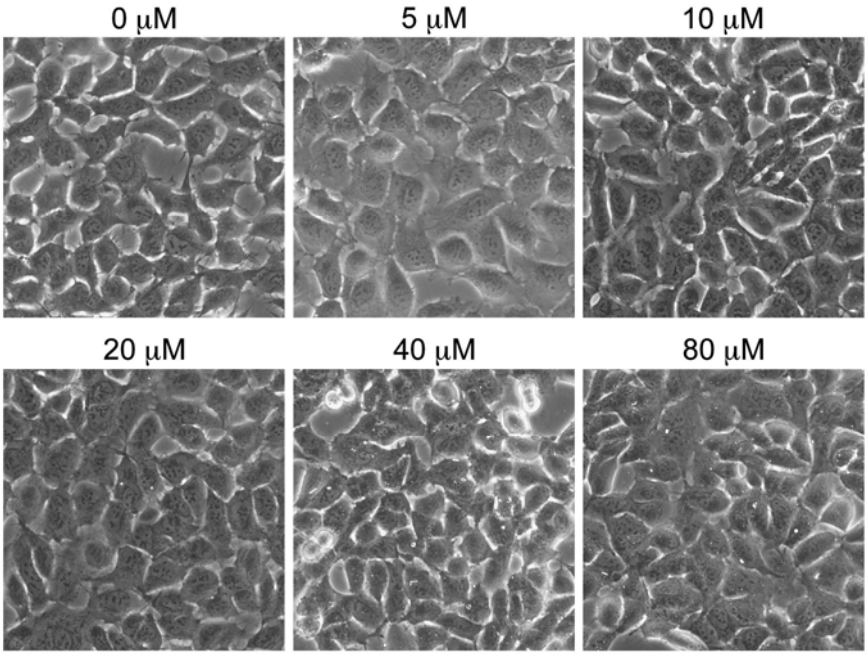


Fig. SB8 Cytotoxicity assay of diyne **3**. HEK293 cells were incubated with DMEM supplemented with FBS (10%), penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}), and diyne **3** (0 to 80 μM) overnight in a 5% CO_2, water-saturated atmosphere. Morphological images of the cells were taken under a phase-contrast microscope equipped with a CCD camera. Cytotoxicity of diyne **3** was rarely observed.

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