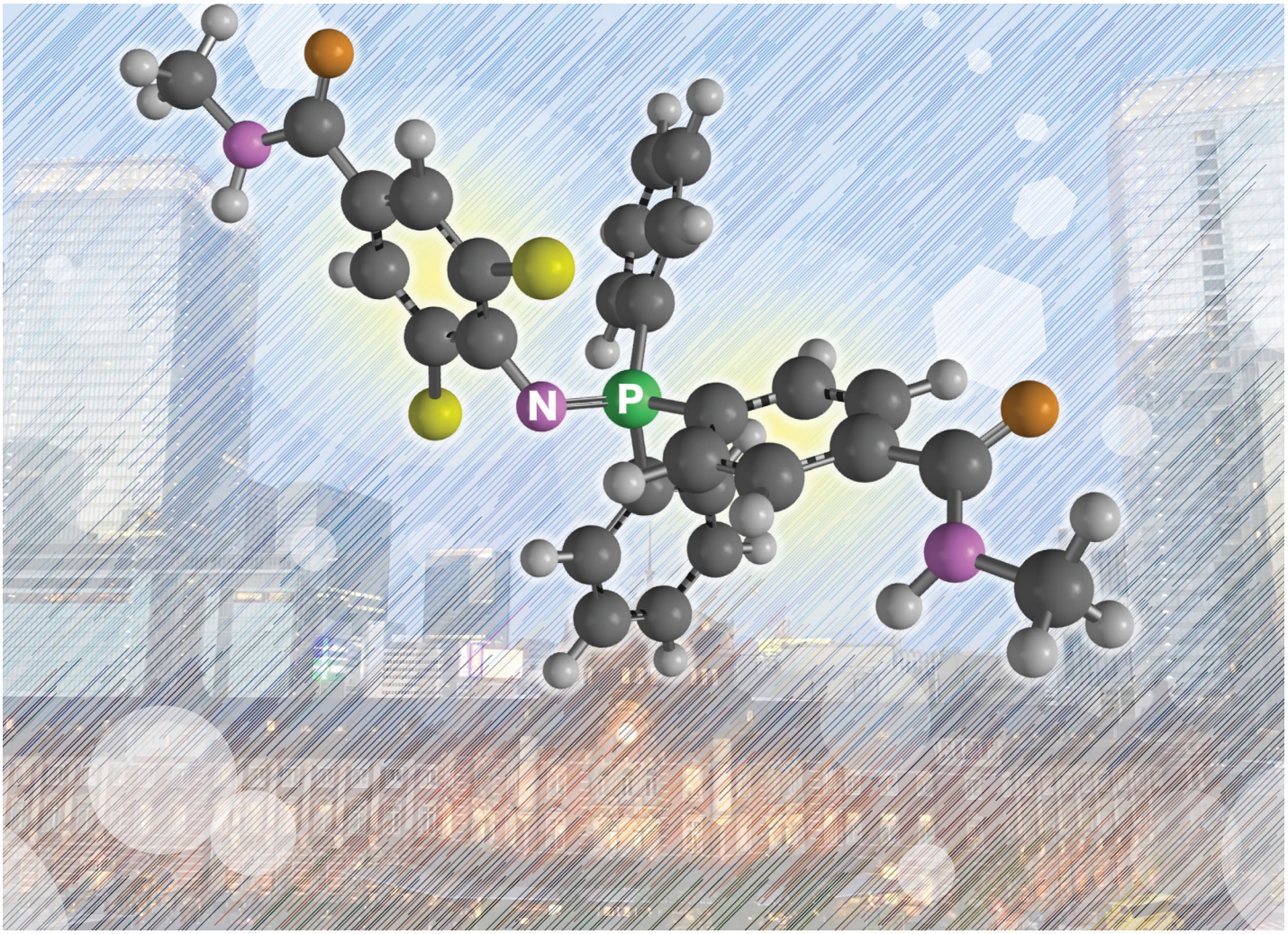

Showcasing research from the group of Dr Suguru Yoshida and Professor Takamitsu Hosoya at the Institute of Biomaterials and Bioengineering, Tokyo Medical and Dental University (TMDU), Japan and the group of Dr Isao Kii at RIKEN, Japan.

Staudinger reaction using 2,6-dichlorophenyl azide derivatives for robust aza-ylide formation applicable to bioconjugation in living cells

Efficient bioconjugation in living cells has been achieved by classic yet new chemistry, just like the retro modern Tokyo station.

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Staudinger reaction using 2,6-dichlorophenyl azide derivatives for robust aza-ylide formation applicable to bioconjugation in living cells†

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Efficient formation of water- and air-stable aza-ylides has been achieved using the Staudinger reaction between electron-deficient aromatic azides such as 2,6-dichlorophenyl azide and triarylphosphines. The reaction proceeds rapidly and has been successfully applied to chemical modification of proteins in living cells.

Click reactions,[1] including copper(I)-catalyzed azide–alkyne cycloaddition (CuAAC)[2] and strain-promoted azide–alkyne cycloaddition (SPAAC),[3] have been recognized as epoch-making methods for reliable conjugations of molecules over a broad range of research fields within chemistry and biology. In particular, click reactions resulting in efficient formation of stable covalent bonds have been widely utilized for chemical modification of biomolecules in chemical biology and drug discovery research.[4] However, several problems using conventional methods, such as non-specific in-cell click labeling by SPAAC, have been reported; thus, a new method is required to address these issues.[5]

Staudinger–Bertozzi ligation using triarylphosphines bearing an *ortho* ester moiety in conjugation with aliphatic azides has emerged as an early bioorthogonal reaction (Fig. 1A).[6] The method forms a robust amide bond and has been demonstrated to be useful for the chemical modification of various biomolecules. Nevertheless, Bertozzi and coworkers developed a SPAAC reaction to achieve a faster and more efficient bioconjugation. Thereafter, a number of methods using cyclooctynes with improved characteristics have been reported.[7] In the course of our recent studies regarding phosphorus chemistry[8] and molecular conjugation chemistry,[9] we revisited the Staudinger reaction between aromatic azides and various phosphines.[8b] These studies gave us an idea of preparing an aza-ylide that would be stable toward hydrolysis and oxidation. We considered that this type of aza-ylide would be useful for chemical modification of biomolecules. Herein, we report a new method for molecular conjugation using the Staudinger reaction to form robust aza-ylides. This chemistry has been found to be applicable to efficient bioconjugation in living cells (Fig. 1B).[10,11]

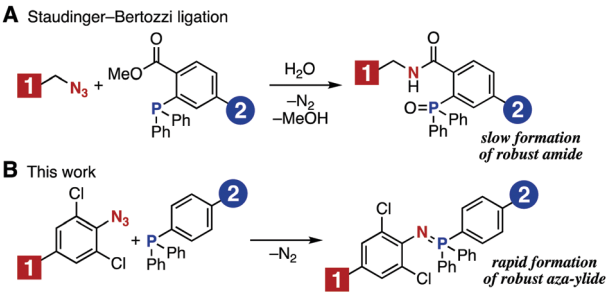

Fig. 1 Molecular conjugation using the Staudinger reaction. (A) Staudinger–Bertozzi ligation. (B) This work, using electron-deficient aromatic azides and triarylphosphines.

After extensive screening of aromatic azides for the Staudinger reaction with triphenylphosphine in the presence of water, we found that 2,6-dichlorophenyl azide was efficiently transformed to the corresponding aza-ylide without the formation of the aniline derivative (Table 1). An initial attempt of the reaction between sterically congested 2,6-diisopropylphenyl azide (**1a**) and triphenylphosphine (**2a**) by stirring the mixture in tetrahydrofuran (THF) and water (v/v = 10/1) at room temperature for 24 h afforded aza-ylide **4a** along with a small amount of side-products (entry 1). When electron-rich aromatic azides **1b–1d** were employed, the yields of anilines **3b–3d**, formed from hydrolysis

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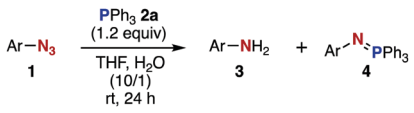
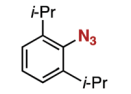
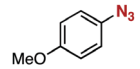
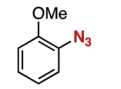
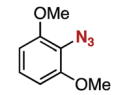
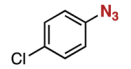
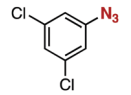
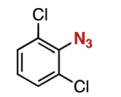
† Electronic supplementary information (ESI) available: Experimental procedures, and characterization for new compounds including NMR spectra. See DOI: 10.1039/c8cc00179k

Table 1 Screening of aromatic azides to form stable aza-ylides using triphenylphosphine (**2a**)



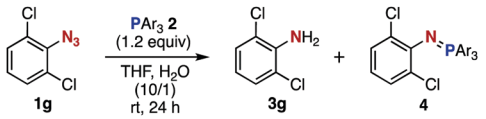
Entry	ArN_3	1	3	Yield of 3[a] (%)	4	Yield of 4[a] (%)
1[b]		**1a**	**3a**	0	**4a**	95
2		**1b**	**3b**	20	**4b**	75
3		**1c**	**3c**	34	**4c**	65
4		**1d**	**3d**	18	**4d**	81
5		**1e**	**3e**	8	**4e**	92
6		**1f**	**3f**	2	**4f**	97
7		**1g**	**3g**	0	**4g**	98 (quant)[c]

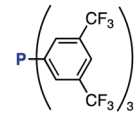
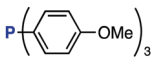
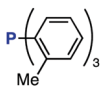
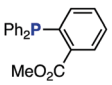
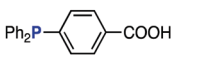
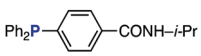
[a] Yields were determined by ^{1}H NMR analysis. [b] A small amount of unknown side-products (*ca.* 4%) were obtained. [c] Isolated yield shown in parentheses.

of aza-ylides **4b–4d**, increased (entries 2–4). In contrast, the formation of anilines was suppressed using electron-deficient aromatic azides such as chloro- and dichlorophenyl azides **1e–1g** (entries 5–7). In particular, the reaction of 2,6-dichlorophenyl azide (**1g**) and triphenylphosphine (**2a**) afforded aza-ylide **4g** quantitatively after purification by silica-gel column chromatography (entry 7). The formation of hydrolyzed product **3g** was not observed, clearly indicating that aza-ylide **4g** was stable toward water and air. In addition, azide **1g** was stable in the presence of an excess amount of *n*-dodecanethiol and in cell lysate, and aza-ylide **4g** remained unchanged in solutions that contained hydrochloric acid, sodium bicarbonate, cysteine, lysine, or tyrosine, demonstrating its remarkable stability.[12]

The efficiency of aza-ylide formation from 2,6-dichlorophenyl azide (**1g**) was greatly affected by the substituents on the phenyl groups of the triarylphosphines (Table 2). Although the reaction between **1g** and electron-deficient phosphine **2b** did not afford aza-ylide **4h** and aniline **3g** was formed instead (entry 1), the quantitative formation of aza-ylide **4i** was observed when electron-rich phosphine **2c** was employed (entry 2). The bulky tri(*o*-tolyl)phosphine (**2d**) did not react with **1g** under the standard conditions (entry 3). Phosphine **2e**, bearing an *ortho* ester moiety, reacted smoothly with **1g** to afford aza-ylide **4k** quantitatively (entry 4). No imine[13] or Staudinger–Bertozzi ligation product[6] was found. While the use of phosphine **2f**, bearing a

Table 2 Screening of triarylphosphines to form stable aza-ylides from 2,6-dichlorophenyl azide (**1g**)



Entry	PAr_3	2	Yield of 3g[a] (%)	4	Yield of 4[a] (%)	Recovery of 1g[a] (%)
1		**2b**	52	**4h**	0	0
2		**2c**	0	**4i**	Quant	0
3		**2d**	0	**4j**	0	97
4		**2e**	0	**4k**	Quant	0
5		**2f**	27	**4l**	69	0
6		**2g**	0	**4m**	99	0

[a] Yields were determined by ^{1}H NMR analysis.

para carboxy group, yielded a significant amount of aniline **3g** *via* hydrolysis of aza-ylide **4l** (entry 5), phosphine **2g**, having a *para* amide moiety, quantitatively afforded aza-ylide **4m**, showing the superior stability of **4m** over **4l** (entry 6). From the kinetic study of the Staudinger reaction between 2,6-dichlorophenyl azide (**1g**) and phosphine **2g** in acetonitrile-d_3, the second-order rate constant was determined to be 0.63 ± 0.02 M^{-1} s^{-1}. This was 250-fold higher than that for the amide formation between benzyl azide and **2e**[6d] and about two-fold higher than that for the SPAAC between benzyl azide and a bicyclo[6.1.0]non-4-yne (BCN) derivative.[7e]

Several competition experiments also demonstrated a rapid Staudinger reaction between 2,6-dichlorophenyl azide (**1g**) and triphenylphosphine (**2a**) (Fig. 2). Treatment of an equimolar mixture of phosphines **2a** and **2e** with azide **1g** predominantly afforded **2a**-derived aza-ylide **4g**, showing that the *ortho* ester moiety of **2e** significantly decreased the reactivity toward azide **1g** (Fig. 2A). The treatment of an equimolar mixture of **1g** and benzyl azide (**5**) with triphenylphosphine (**2a**) predominantly afforded aza-ylide **4g**, demonstrating a remarkable reactivity of electron-deficient aromatic azide **1g** toward phosphines (Fig. 2B). The treatment of an equimolar mixture of **2a** and BCN derivative **8** with azide **1g** in methanol afforded almost equal amounts of aza-ylide **4g** and triazole **9**, indicating that the Staudinger reaction proceeded as fast as the SPAAC reaction[7e] (Fig. 2C).

Further competition experiments using an equimolar mixture of phosphine **2a** and dibenzo-fused cyclooctyne **10** exhibited a unique orthogonality (Fig. 3). While the Staudinger reaction of azide **1g** with **2a** proceeded significantly faster than the SPAAC reaction of **1g** with **10** (Fig. 3A-1), benzyl azide (**5**) exclusively

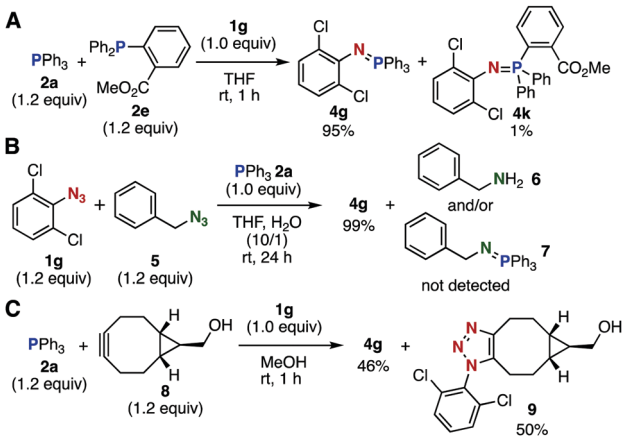


Fig. 2 Competition experiments. (A) Phosphines. (B) Azides. (C) Staudinger reaction *vs.* SPAAC reaction. Isolated yields are shown.

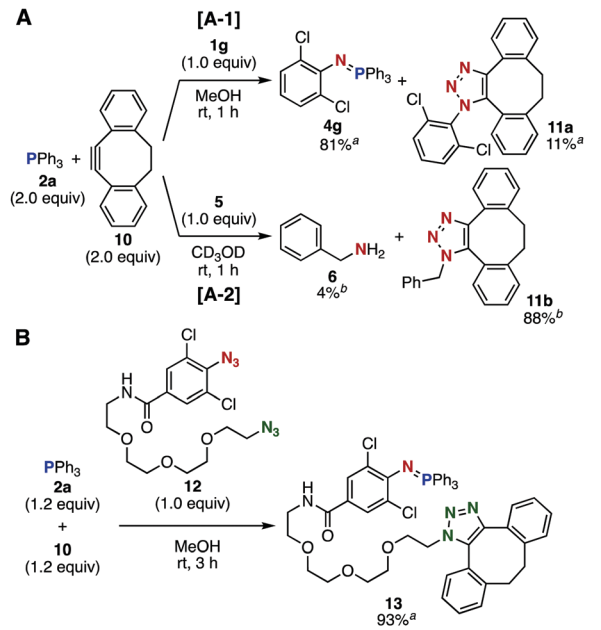


Fig. 3 Selective conjugations. (A) Reactions of an equimolar mixture of phosphine **2a** and cyclooctyne **10** with azide **1g** or **5**. (B) Reaction of diazide **12** with an equimolar mixture of **2a** and **10**. [a] Isolated yields are shown. [b] Yields were determined by ^{1}H NMR analysis.

reacted with cyclooctyne **10** to afford triazole **11b** along with a small amount of Staudinger reaction-derived product **6** (Fig. 3A-2). This orthogonality between Staudinger and SPAAC reactions enabled simultaneous bisconjugation in a site-selective manner using diazide **12** bearing 2,6-dichlorophenyl azide and alkyl azide moieties (Fig. 3B). Thus, the treatment of an equimolar mixture of phosphine **2a** and cyclooctyne **10** with diazide **12** afforded a three-component coupled product **13** in high yield. This result indicated that the Staudinger reaction with **2a** and SPAAC reaction with **10** proceeded selectively at the 2,6-dichlorophenyl azide and alkyl azide sites of diazide **12**, respectively, which served as an efficient hinge molecule to conjugate two different types of azidophiles.

The formation of a stable aza-ylide by the Staudinger reaction between 2,6-dichlorophenyl azide and triarylphosphine

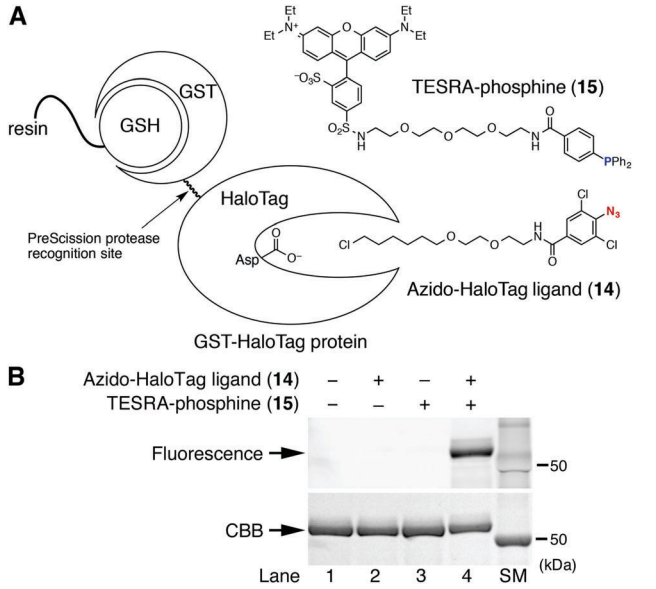


Fig. 4 Chemical modification of an azido-protein using the Staudinger reaction. (A) GST-HaloTag protein bound on GSH-resin, azido-HaloTag ligand (**14**), and TESRA-phosphine (**15**). (B) SDS-PAGE analysis of the labeled GST-HaloTag proteins eluted from the resin. The gel was scanned using a fluorescence image analyzer and then stained with Coomassie brilliant blue (CBB). SM indicates the size marker lane.

was applied to the chemical modification of biomolecules (Fig. 4). According to the previous report,[7f] an azido-protein was prepared by conjugating GST-fused HaloTag protein (GST-HaloTag) with the azido-HaloTag ligand (**14**) on a GSH-conjugated resin,[12,14] followed by the treatment with fluorescent TESRA-phosphine (**15**) (Fig. 4A). The following SDS-PAGE analysis showed that TESRA-labeled GST-HaloTag protein (51 kDa) was successfully prepared (Fig. 4B, lane 4).[12] This result indicated that the aza-ylide formed from the Staudinger reaction was sufficiently stable under bioconjugation conditions, demonstrating the bioorthogonality of this method.[12,14]

The novel Staudinger ligation was also applicable to chemical modification of proteins in living cells (Fig. 5). For example, cell surface-specific fluorescent labeling was achieved by the expression of transmembrane domain-fused HaloTag protein on the cell surface, in which HaloTag is present outside the cells, followed by the treatment with azido-HaloTag ligand (**14**) and TESRA-phosphine (**15**) (Fig. 5A). Notably, our Staudinger ligation method was also effective for fluorescent labeling of HaloTag fused with NUP133 nuclear pore complex protein (Fig. 5B). This result indicated that this method could be used for chemical modification of intracellular biomolecules. Indeed, compared with the fluorescent labeling method using SPAAC modification with a dibenzo-fused cyclooctyne possessing a TESRA moiety,[12] our Staudinger ligation method showed a superior result in terms of labeling efficiency inside the cells.

In summary, we have demonstrated that the Staudinger reaction of 2,6-dichlorophenyl azide derivatives with triarylphosphines proceeds rapidly to form robust aza-ylides. The method allows for the efficient chemical modification of proteins in living cells.

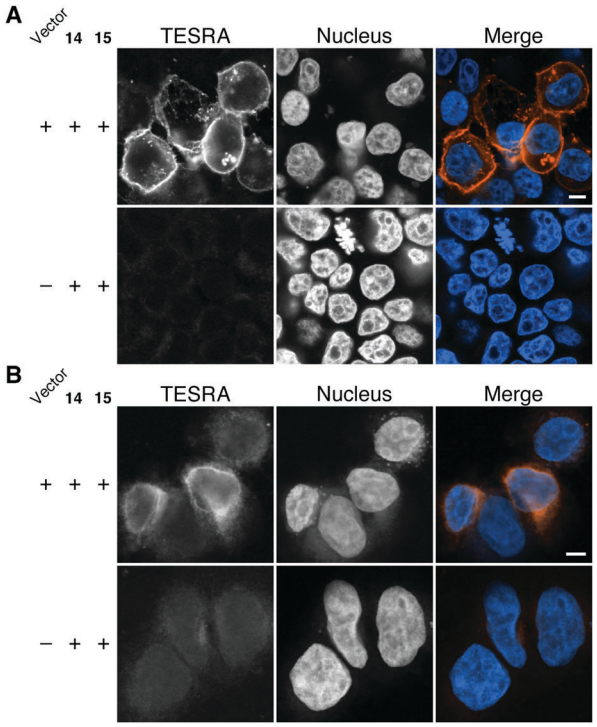


Fig. 5 Fluorescent labeling of living cells expressed with HaloTag-fused proteins by incubation with 10 μM of azido-HaloTag ligand (**14**) for 30 min at 37 °C, followed by incubation with 1 μM of TESRA-phosphine (**15**) for 30 min at 37 °C. (A) HEK293 cells with HaloTag protein on the cell surface outside the cells. (B) HEK293 cells with HaloTag protein on the nucleus inside the cells. Vector (+) indicates the expression of the HaloTag fusion proteins, and (–) indicates no expression. Scale bar, 5 μm.

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Conflicts of interest

There are no conflicts to declare.

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